Novel Diagnostic Approaches for Genetic and Environmental Sources of Mitochondrial Dysfunction

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Technical Abstract

With cardiovascular disease, diabetes mellitus, and neurodegenerative conditions on the rise, understanding their pathogenesis is paramount to tackling this public health crisis. Current research indicates that the primary cause of these diseases is mitochondrial dysfunction in the affected patients. While genetics plays a role in these conditions, lifestyle choices and exposure to toxins also significantly contribute to their development. Unfortunately, early-stage diagnosis can be difficult due to overlapping symptoms with other diseases. Developing innovative therapies that can prevent or reverse the deterioration of metabolic dysfunctions is critical to establishing early intervention. My research focused on investigating molecular targets linked with Friedrich's Ataxia, an inherited metabolic disorder, through conducting functional in-vitro studies using human-derived cell samples, as well as developing inventive animal models created via Xenopus laevis tadpoles to evaluate the effects of environmental stressors. My investigations have uncovered promising treatment options that improve mitochondrial function, mitigate oxidative stress, and elucidate critical mechanisms involved in environmentally induced disruptions to mitochondria.

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General Audience Abstract

Metabolic dysfunction is a widespread health issue that affects millions of individuals each day. Its associated disorders, such as cardiovascular disease, diabetes, and neurodegenerative conditions, are rising due to various factors ranging from genetic predispositions to environmental and lifestyle-related risks. Therefore, there's an urgent need to identify this disorder early on and develop innovative treatment options. Considering this growing public health concern, it has become imperative to establish new methods for detecting metabolic dysfunction at its nascent stage while also exploring potential therapeutic interventions. Our research utilized cells derived from affected patients and animal models in devising novel approaches toward understanding the molecular mechanisms underpinning metabolic dysfunction. Our findings revealed several pathways and molecular targets contributing significantly to this condition, which could effectively be leveraged to develop targeted therapeutic strategies to combat its effects. Expanding our knowledge base will enable us to stay updated with emerging insights on treating metabolic dysfunction effectively while substantially improving patient outcomes.

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Chapter I: Introduction

The mitochondrion, an essential organelle for eukaryotic cells, is believed to have originated from the symbiosis between a bacterium and a eukaryotic cell. This evolutionary process led to the development of over 1500 specialized proteins, which enhance intra-organelle efficiency and provide cellular response mechanisms to various stimuli [1, 2]. Several molecules such as reactive oxygen species, calcium ions (Ca2+), oxygen (O2), and compounds like monophosphate and diphosphate(di-AMP)/triphosphates/tri adenosines/flavin adenine dinucleotide play pivotal roles in regulating these responses by acting as signals for triggering intercellular adaptations. In a healthy state, mitochondria work in an interconnected network of dedicated counterparts. A dynamic exchange of nutrients, metabolites, DNA, and ions efficiently supports the organism's energetic demand. After the healthy mitochondrion has fulfilled its purpose or shows signs of malfunction or reduced metabolic status, intracellular signals induce the process of mitophagy and deliver it to lysosomes for degradation, ensuring optimal efficiency in the cell's metabolism. The regulation of these critical characteristics ultimately culminates in the severity of response to pathological conditions [3, 4]. In development, disease, or increased exertion, mitochondria may undergo biogenesis. Increasing the number of mitochondria within the cell provides optimal reactivity and a reduced load for each organelle. However, toxic exposure or disease states can tilt the sensitive balance of energy production in the mitochondria, resulting in detrimental effects on cells leading to stress signal pathway responses triggered by misfolded protein aggregates, among other factors causing mitochondrial dysfunction implicated in many diseases like Alzheimer's Disease or Parkinson's Disease, diabetes, and cardiovascular disease

among others. Historically, treating metabolic-related maladaptation has been a broad-brush approach, prioritizing the symptoms rather than the root cause [5]. When considering the bioenergetic differences between various tissues and the expressed or mutated mitochondrial DNA (mtDNA), a dynamic complexity arises. Tissue-specific obligations promote the required mtDNA expression, ensuring the optimal mitochondrial machinery and processes are employed [6, 7].

Heritable mutations in the expressed mtDNA can cause dissociation or inexpression of these vital mechanistic properties. The locality of its existence within the organism gives way to structural variations, mechanisms, and susceptibility to disease or toxic states. For example, neuronal-specific mitochondrial structuring increases the susceptibility to oxidative damage, especially from neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) [8]. Further ramifying the broad-brush approach to medical intervention is the increased exposure to endogenous or exogenous toxins and environmental pollutants. Pathological conditions in bioenergetics that result from genetically transcribed disruption can also be expressed similarly due to environmentally implicated, non-transcriptive consequences [9]. With consumer-based toxins being voluntarily used and adversely affecting metabolic health in present populations, a common understanding is vital now more than ever [10, 11].

We aim to evaluate the overarching hypothesis of whether the various sources of mitochondrial dysfunction result in the same fundamental characteristics: a disruption to mitochondrial energy production and formation. The varied spectrums of mitochondrial pathologies/underlying bioenergetic complications inflict various health issues, offering longevity challenges and susceptibility to many other diseases [3]. In the following chapter, we will review the current literature on how the etiology of mitochondrial disease needs to be identified. To understand how mitochondria modulate various human disorders and lifespan, this chapter delves

into the essential biodynamics that can be affected under different genetic predispositions and environmental exposures. The subsequent chapters provide a comprehensive understanding and new perspectives on the various causes of mitochondrial dysfunction.

A methodical approach is necessary to distinguish this unique physical characteristic from disease. Chapter three delves deeper into mitochondrial health using techniques that monitor oxidative phosphorylation, fluorescence signals, gene expression, and other bioenergetic indicators. These measures evaluated the effectiveness of various treatments and cellular interventions for modulating mitochondrial disease or genetic dysfunction. Nevertheless, their clinical applicability could have been improved due to the limited use of an in-vitro model in obtaining these results. Nonetheless, validating novel therapeutic methods for treating diseases carries significant value in comprehending mitochondrial malfunctioning as it presents itself across various associated illnesses.

The lab of Dr. David Brown, which was initially developed to study cardiac physiology and pharmacology, shifted toward the vital energy source in the heart, the mitochondria. This shift was driven by recognizing mitochondria's critical role in cellular energy production and their involvement in numerous human diseases, including metabolic disorders. To investigate the role of mitochondria in the human condition, the lab employed a multifaceted research approach that integrated molecular biology, biochemistry, and physiology with cutting-edge technologies such as functional cardiology systems, high-resolution respirometry, metabolomics, pathway analysis, sequencing, and next-generation microscopy. This comprehensive approach allowed for a detailed analysis of mitochondrial function and its impact on cellular metabolism in normal and disease states. Furthermore, our laboratory's experimental approach involves a meticulous and structured process encompassing in vitro investigations, analyses of intact hearts, and human samples. We

aim to determine the plausibility of identifying specific ailments associated with mitochondrial diseases by testing various hypotheses. This rigorous assessment has successfully uncovered emerging understandings regarding mitochondrial function across different physiological and pathological scenarios. This has led us to develop new therapeutic strategies to mitigate the impacts caused by mitochondrial disorders on human health: expanding our knowledge base about them while exploring novel pharmacological targets for their treatment.

The following chapter, chapter four, builds upon the previously established investigative tools to delve into a more comprehensive in-vivo model. These studies focus on the highly energetic and complex tissue, the brain, to examine a source of mitochondrial dysfunction that affects not only individuals with heritable diseases but also those who are otherwise healthy. This section presents findings from research conducted in Dr. Christopher Thompson's laboratory, a researcher renowned for his expertise in neuroendocrinology. The lab of Dr. Thompson utilized the *Xenopus laevis* tadpoles to monitor environmental contaminants on the disruption of the developing endocrine system, particularly Triclosan (TCS) and lead. The lab has since expanded its focus to encompass the bioenergetic source of this developmental disruption. Using advanced imaging techniques and in-vivo models, we demonstrate that TCS exposure disrupts mitochondrial function and impairs brain development. This research utilized tools from historically in-vitro cell models and adapted them to the more clinically relevant in-vivo model using the Xenopus laevis. We test the hypothesis that exposure to TCS results in mitochondrial dysfunction leading to impaired energy metabolism in developing animals. These studies provide a platform for significant advancement in understanding the impact of environmental toxicants on mitochondrial function and neurological development. Moreover, this model can be an essential tool for future

pre-clinical studies that aim to understand the underlying mechanisms and sources of mitochondrial dysfunction.

The final chapter, chapter five, delves into the potential implementation of mitochondrial function in clinical settings using our innovative in-vivo platform. Notably, this model can support the development of therapeutic approaches such as small molecule therapies and cell-based interventions to enhance their efficacy in treating mitochondrial dysfunction-related diseases. To broaden our horizons further and improve treatment outcomes significantly, it is essential to incorporate other pre-clinical models alongside these treatments to understand mitochondrial functions' underlying mechanisms comprehensively. By expanding our comprehension through collaborative efforts among various fields of study, we could unearth groundbreaking possibilities that would enable us to treat maladies associated with dysfunctional mitochondria effectively. Therefore, academic expansion via cooperation has immense potential for advancing medical science's frontiers toward effective solutions against related diseases derived from malfunctioning mitochondria.

Specific Aims

- Explore the causes and effects of mitochondrial dysfunction.
- Design treatments to target specific mechanisms related to mitochondrial diseases.
- Identify mechanisms by which environmental toxicants, such as TCS, disrupt mitochondrial function and impair brain development.
- Develop an in-vivo model utilizing *Xenopus laevis* to understand better the impact of environmental toxicants on mitochondrial function and neurological development.

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Chapter II: The Environmental and Mutagenetic Implications on Mitochondrial Form and Function- Literature Review

Abstract

Mitochondria are essential organelles in the cell that generates energy by oxidative phosphorylation. Defects in mitochondrial energy production (i.e., function) have been implicated in various diseases, including diabetes, cardiovascular disease, and neurodegenerative diseases such as Parkinson's and Alzheimer's. Both inherited mutations in mtDNA, and environmental factors, such as nutrient deficiencies, toxic chemicals, and stress, can alter the structure and function of mitochondria. These environmental stressors can further disrupt predisposed or mutated mitochondria to cause and further the progression of the disease. Therefore, understanding the molecular mechanisms underlying mitochondrial dysfunction can provide insights into how these pathways can be targeted to prevent and treat conditions associated with mitochondrial dysfunction.

Introduction

The production of adenosine triphosphate (ATP) through oxidative phosphorylation is a vital process that requires the maintenance of homeostasis within both the mitochondrial network and internal machinery. However, when this delicate balance is disrupted, the result is a failure to generate sufficient amounts of ATP. Failure to efficiently produce ATP is a fundamental component of mitochondrial dysfunction, which has far-reaching consequences and has been

linked to numerous pathological conditions such as metabolic disorders and neurodegenerative diseases. Metabolic disorders can be characterized by an abnormal metabolism that affects various bodily processes, including glucose regulation, lipid metabolism, and energy production. Moreover, disturbances in mitochondrial function have also been associated with several conditions, such as Parkinson's disease (PD), Alzheimer's disease (AD), Friedreich's ataxia (FRDA), and Huntington's disease (HD). Maintaining proper homeostasis within mitochondria is crucial for cellular survival since alterations in their functionality could lead to insufficient energy generation and severe health complications.

Disruption of mitochondrial function due to environmental and genetic factors can ultimately result in cellular death and an increase in pathological conditions. Mitochondrial disruption can be attributed to both exogenous and endogenous factors. Exogenously, exposure to environmental toxins like pollutants, chemicals, and heavy metals damages mitochondrial networking and processing, thus affecting ATP production. Inadequate ATP production subsequently increases unmanaged ROS production within cells, which could lead to cell death through apoptosis [1]. Conversely, genetic disorders that modify the metabolic dynamics or structure of mitochondria result in comparable states of mitochondrial dysfunction [2]. Therefore, it is imperative to comprehend the mechanisms that uphold mitochondrial energy production and identify how heritable and environmentally induced damage disrupts them.

Whether the source for mitochondrial dysfunction is heritably or environmentally linked, the current therapeutic approaches prioritize symptomatic improvements over the etiology of the derived illness. For example, treatment options may include dopamine replacement therapy to manage PD symptoms, insulin injections for diabetes-related blood sugar level regulation, and

statins to reduce cholesterol in cardiovascular diseases [2–4]. But while current treatments may provide some relief from symptoms associated with these debilitating conditions, they fail to address the root cause of mitochondrial dysfunction. We must understand the interdependent mechanisms behind mitochondrial disruption to develop effective therapies that can restore cellular homeostasis and repair energy inefficiencies at their source. By doing so, we can identify potential therapeutic targets for modulation through drugs or genetic approaches that support mitochondrial dynamics, function, and biogenesis, offering new hope for those suffering from chronic diseases linked to impaired mitochondria-energy metabolism coupling.

A complex interdependent system of critical mechanisms maintains mitochondrial homeostasis, including mitophagy, mitochondrial fusion and fission, biogenesis, and oxidative phosphorylation. These processes are essential for maintaining proper mitochondrial energy production within cells. Mitophagy is an important mechanism in this process, which removes damaged or faulty mitochondria from the cell. On the other hand, fusion regulates energy production by ensuring connectivity between the various networks of mitochondria inside a cell and promoting efficient cellular respiration. Fission is another crucial mechanism that reduces potential pathogens' spread through mitochondrial compartments and prevents harmful cell mutations. The interplay between these dynamic processes ensures effective intra-organelle communication and overall bioenergetics balance to maintain cellular health. Several genes have been identified as accountable for regulating formational processes and simultaneously affecting their functionality during conditions of cellular stress. Therefore, strategies that modulate formational processes could provide an avenue for restoring mitochondrial function and rescuing cells from dysfunction-induced death.

Understanding molecular and cellular signals and the environmental and heritable factors that affect them is crucial for developing effective therapeutic strategies to repair damaged mitochondria. Unraveling the complexities of mitochondrial dysfunction resulting from these factors is essential in understanding the pathophysiology of various diseases and improving patient outcomes through restoring mitochondrial function. Furthermore, targeted therapies that aim to replace damaged or mutated mitochondrial DNA (mtDNA) hold promise for addressing heritable mitochondrial dysfunction. Therefore, in-depth knowledge of the complex mechanisms regulating mitochondrial homeostasis and their role in various diseases can aid researchers' quest to develop novel drug treatments and therapies that target mitochondrial dysfunctions.

Physiological and Biochemical Changes in Disease States

A vicious cycle can be initiated when respiration or remodeling cannot adapt to changing metabolic demands. Remodeling processes allow tissues to adapt to changing environmental conditions and physiological needs but become targets to mutagenic and environmental stressors that can disrupt the homeostasis of mitochondrial function. In heritable disease states where defective mitochondrial DNA (mtDNA) can impair mitochondrial function, altered oxidative phosphorylation efficiency can increase susceptibility to environmental factors, further promoting ROS accumulation and mitochondrial DNA damage, leading to disease progression. This results in a vicious cycle of mitochondrial damage that is exacerbated in the setting of impaired mitochondrial dynamics.

Mitochondrial dysfunction is a complicated process affecting energy production through the electron transport chain (ETC). The ETC consists of 13 subunits controlled by 37 mitochondrial DNA genes, and it plays an essential role in cellular respiration. However, mutations or maladaptation may disturb ETC's function leading to severe ailments such as Friedreich ataxia, Neuropathy target esterase deficiency, Leber hereditary optic neuropathy, and Mitochondrial myopathy with varying clinical symptoms. These disorders involve altered protein expression and function resulting from genetic defects affecting the mtDNA-encoded ETC subunits essential for efficient aerobic metabolism. See Table 1 for other examples of heritable mitochondrial diseases and the implicated mtDNA causes. The physical loss of critical proteins may not be immediately recognizable or detrimental to the organelle's function in a homeostatic environment. Still, it can accelerate mitochondrial damage and compromise energy production during periods of stress. Under these conditions, the cell must increase mitochondrial fusion and fission to maintain proper energy balance. In the state of fission, the duplication of mutated mitochondria occurs as the organelle splits into two separate mitochondria. This increases the expression of mutated mitochondria in the cytosol and can lead to the release of pro-apoptotic signals, such as cytochrome-c, from the outer membrane that induces cell death. These mutated mitochondria can transfer through macrophage or extracellular vesicle-related transfer to neighboring, thus spreading the mutation following cells. An increase in the expression of mutated mitochondria can also occur by mitochondrial fusion, where an intact organelle acquires a portion of another donor molecule to form a new mitochondrion containing both genomes. This can lead to the formation of unstable intermediates that eventually collapse under the weight of their genetic material and subsequently causes the release of pro-aprotic signals [5–8]. (see Figure 1 below).

Although it is important to consider mechanisms that can enhance cell survival in the face of mitochondrial dysfunction, these processes can also promote accelerated disease progression by stimulating the release of cytotoxic factors capable of damaging adjacent cells [8]. In the context of neurodegenerative diseases such as Alzheimer's and Parkinson's, this process can be detrimental because it can result in the loss of neurons that produce critical neurotransmitters that allow the brain to communicate with other parts of the body. Increasing evidence suggests that dysfunctional mitochondrial networks can contribute to neural toxicity by interfering with neurotransmitter production and their ability to communicate with downstream signaling pathways contributing to disease pathogenesis. For example, impaired activation of tyrosine hydroxylase (TH) in dopaminergic neurons of the substantia nigra is associated with the development of Parkinson's disease (PD). During PD progression, this protein is phosphorylated at multiple sites by enzymes such as glycogen synthase kinase-3β (GSK-3β) that trigger mitochondrial dysfunction and promote the production of toxic free radicals that contribute to the degeneration of these cells [9, 10]. Similar mechanisms may also contribute to the death of dopaminergic neurons observed in individuals with Alzheimer's disease [11, 12]. Indeed, a recent study has demonstrated that defective activation of the enzyme monoacylglycerol lipase (MAGL) can contribute to the development of AD by reducing cholesterol esters and activating the inflammation response in the brain, thus mimicking a PD state of dysregulation [13].

Before the dysregulated states, mitochondria may utilize the process of mitophagy to selectively eliminate defective mitochondria and thereby restore homeostasis in the cell. However, aberrant mitophagy is associated with neurodegenerative disorders, PD, AD, and Amyotrophic lateral sclerosis (ALS). The downregulation of mitophagy has been shown to contribute to the development of these diseases by increasing the survival of mutant mitochondrial populations.

This downregulation is due to several mediators inhibited in the diseased state, including parkin and PTEN-induced putative kinase 1 (PINK1). In the case of parkin/PINK1 dysregulated mitophagy, this is thought to be due to the induction of reactive oxygen species (ROS) within the cell that inhibits the activation of the ubiquitination system and prevents the binding of parkin to damaged mitochondria. As a result, these mutated mitochondria accumulate within the cell and promote the production of pathogenic signaling molecules that can lead to neuronal death. Mitochondrial signaling is crucial in maintaining cellular homeostasis and preventing human diseases. However, external stressors such as toxins can disrupt these intricate mechanisms, resulting in mitochondrial dysfunction and its associated ailments.

Environmentally Imposed Mitochondrial Maladaptation and Mutations

The genetic deficiencies and mutations mentioned earlier as causative factors of maladaptive changes within cells could also manifest themselves through contemporary lifestyle aspects. Cell mitochondria dysfunction resulting from various environmental stresses like excessive physical exertion, toxicant exposures, prolonged exposure to stress, chronic infections, poor nutrition and diet choices, as well as smoking or alcohol abuse are potential agents contributing towards pathological disorders. The contributing pathological conditions can also directly affect the expression of mtDNA and histone tail modifications predisposing the cell to develop deleterious mutations during periods of mitochondrial distress. External environmental stressors can exogenously aggravate internal cellular mechanisms and mitochondrial function disturbances, leading to potential disease development. Mutagenic stress alters mtDNA by

changing the protein-coding sequences and non-coding regions, decreasing mitochondrial functioning.

Similarly, environmental stresses such as hypoxia and oxidative stress can increase the production of ROS that can damage mtDNA and lead to further decreases in mitochondrial respiratory efficiency [14]. Prolonged exposure to these environmental conditions can alter the epigenetic state of nuclear DNA, resulting in gene dysregulation and increased mutation frequency [15]. Mutations that affect the stability and normal function of proteins also contribute to the pathogenesis of various mitochondrial diseases. In addition to increasing mutagenesis, environmental factors influence cellular repair pathways. Various environmental factors can also affect the compensatory mechanisms of fission, fusion, and mitophagy, contributing to the accumulation or subsequent damage of malfunctioning mitochondria, as previously discussed. For example, alterations in mitochondrial calcium handling caused by iron overload or oxidative stress can result in abnormal fission dynamics that lead to a loss of membrane potential and accumulation of dysfunctional mitochondria [16, 17]. Excess mitochondrial ROS production and impaired antioxidant defense mechanisms may also contribute to a diminished capacity for mitophagy [18]. Taken together, alterations in various mitochondrial functions are directly attributable to the effects of both environmental and genetic factors on the maintenance of mitochondrial homeostasis.

The mitochondrial dynamics in a postmitotic state dictate the degree of neurogenesis and differentiation that occurs in the developing brain. The mitochondria are directly impacted by the environmental toxin triclosan, a compound found in many household items such as cleaning agents, toiletries, and apparel. Where triclosan is seen to disrupt the natural flow of electrons within the mitochondria and alter the electrochemical gradient. Triclosan's ability to disrupt this gradient leads to misfolding of proteins and improper mitochondrial tethering to the endoplasmic reticulum,

causing increased calcium uptake/ overload and subsequent oxidative stress Field [19]. The specific mechanisms and disrupted processes are further investigated in chapter four. The impact of pollutants on the development of neuropathology in the prenatal period adds further complexity. The severity may depend on the amount and duration of exposure and genetic factors modulating susceptibility.

Refining neurodevelopmental processes through developmental experience mainly depends on a functional repertoire of mitochondria present in neurons throughout development. The more mitochondrial activity, the more neurons are made, and the greater the potential for neural development to occur [19]. These physiological maladaptations in the setting of the prenatal developing brain have been seen to lead to learning disorders in rodents and similar impairments in human populations exposed to similar levels of contamination [20]. Therefore, it is proposed that alterations in mitochondrial function and mtDNA may play an essential role in the etiology of autism spectrum disorders (ASD) in children [19]. Given the chronic nature of mitochondrial diseases and deficits from the maternal environment during gestation, it is estimated that changes in mitochondrial function likely begin at least during the pre-conception period or even earlier and have adverse consequences on the health and behavior of the brain [21]. Therefore, continued awareness and understanding of the mechanisms involved in the damage caused by environmental factors and their impact on the function of mitochondria is essential to develop more effective strategies for treating and preventing neurological and metabolic diseases. See Table 2 for a list of environmental insults and toxins, the associated diseases, and mitochondrial implications.

Therapies and Treatments

Despite considerable progress in understanding the pathogenic mechanisms involved in developing mitochondrial disease, there is still much left to learn before therapeutic strategies for these diseases can be created. Due to the complex nature of the pathogenic mechanisms involved and the varied clinical presentations of mitochondrial diseases, developing effective treatments for most forms of mitochondrial disease has been challenging. However, new methods of identifying the proteins encoded by the mtDNA and developing better assays for diagnosing mitochondrial disease promise to speed up the search for effective therapies for these diseases [22, 23]. In a recent study, Marshall et al. used sequencing techniques and the published data from the Mitomap to identify mutations that affect several crucial components of the electron transport chain in the mtDNA of humans. Approaches such as these can play an essential role in identifying novel therapeutic targets for mitochondrial disease.

Furthermore, researchers are hopeful that advances in gene editing technology will eventually allow researchers to 'correct' disease-causing mutations in vitro so they can be reintroduced into diseased tissues in the body [24, 25]. Although some of these approaches hold great promise, significant challenges must be addressed before these treatments can be widely available to patients. For instance, one of the biggest challenges lies in developing methods to efficiently deliver therapeutic molecules to affected tissues without damaging surrounding healthy cells and in developing drugs that act specifically on the mitochondrial genome. Despite these challenges, a growing body of evidence suggests that some current therapeutics used to treat other conditions may also be beneficial in treating mitochondrial disease. For example, some compounds

commonly used to treat neurodegenerative disorders such as Alzheimer's and Parkinson's have also improved mitochondrial function in various animal models of mitochondrial disease [26].

Nutrition and diet have also been shown to modulate mitochondrial function and treat mitochondrial diseases. Several dietary compounds (e.g., alpha-lipoic acid, coenzyme Q, N-acetylcysteine, etc.) have been shown to stimulate mitochondrial regeneration [27–29]. These diet-derived compounds have been seen to ameliorate the symptoms of several mitochondrial diseases, including ALS, Barth syndrome, Leigh syndrome, and MELAS [28, 30–33]. More recently, it has also been shown that a ketogenic diet can alleviate symptoms in mouse models of Alzheimer's and Huntington's disease by boosting mitochondrial biogenesis and metabolism [34–36]. Diet can also be essential in preventing mitochondrial dysfunction caused by genetic and environmental risk factors. Studies have shown that a diet rich in unsaturated fatty acids reduces the accumulation of toxic metabolites and improves energy production in the mitochondria [37]. In addition, a growing body of evidence suggests that various forms of fasting may be valuable tools in treating mitochondrial disease [38, 39]. This has led researchers to suggest that caloric restriction may have therapeutic potential in patients with mitochondrial disease.

Finally, recent research has highlighted the potential role of epigenetic mechanisms in modulating mitochondrial gene expression and activity [40, 41]. Indeed, it has recently been shown that changes in the expression of specific proteins in the epigenome can control the activity of genes associated with mitochondrial metabolism, thereby influencing the synthesis of key enzymes involved in oxidative phosphorylation and the respiratory chain complex I. These recent findings have raised the possibility that epigenetic therapies may one day have clinical applications in treating mitochondrial disease. Taken together, these findings suggest that the future treatment of

mitochondrial disease will likely involve the development of novel therapies that target the mitochondria directly and/or the epigenome.

Conclusion

Mitochondrial dysfunction has been linked to many diseases and conditions, such as cardiovascular and neurodegenerative disorders and aging. We hypothesize that the various sources resulting in mitochondrial dysfunction affect the same fundamental characteristics, disrupting mitochondrial energy production and formation. Given the heterogeneity in symptoms and underlying causes among individuals with mitochondrial disease, there is no universal approach to treatment. Genetic factors, lifestyle choices, and environmental exposures contribute significantly to the onset of associated conditions. However, ongoing research on endogenous mechanisms responsible for inducing mitochondrial dysfunction and innovative therapeutic interventions offer a promising avenue for tackling these debilitating ailments. The success of these novel treatments hinges largely upon interdisciplinary collaboration among scientists toward comprehending the intricacies involved in mitochondrial disorders leading to the development of more efficacious treatment options.

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Figures

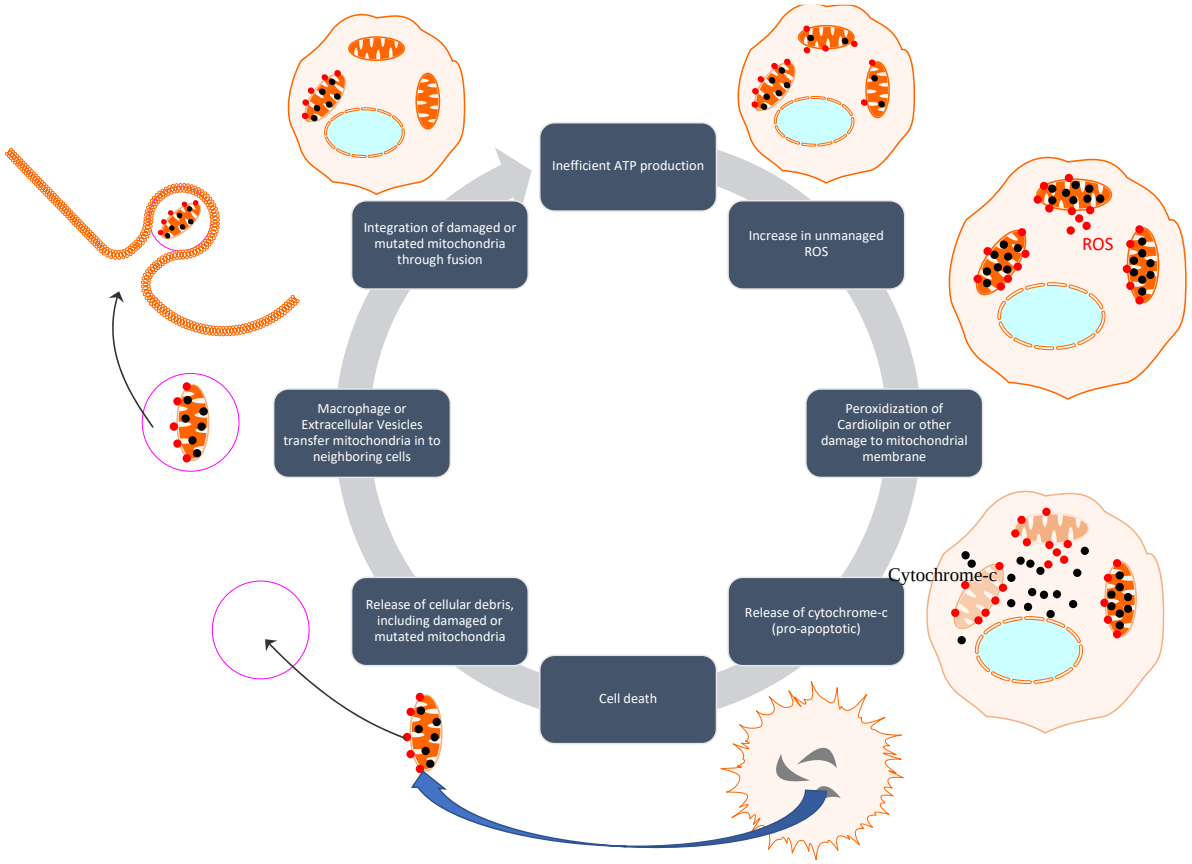

Figure 1: Mutated and Damaged Mitochondria Proliferate in states of Disequilibrium and inefficiency. The vicious cycle of mitochondrial dysfunction initiated by inefficient production of ATP results in unmanaged reactive oxygen species (ROS) and damage to the mitochondrial DNA (mtDNA).

Tables

Table 1: Mitochondria-associated heritable diseases and dysfunctions, their related symptoms, the critical mutations or deficits expressed, and the prevalence.

Mitochondrial Disease	Symptoms	Mutation	Prevalence	Relevant Publications
Friedreich's ataxia	Progressive gait, limb ataxia; dysarthria, areflexia, cardiomyopathy; diabetes mellitus, and secondary skeletal abnormalities.	Excess repeats (>~500 copies) of GAA in intron 1 of the FXN gene	Estimated 1 in 30,000 newborns	[42–44]

Leigh Syndrome	Progressive neurodegenerative disorder, seizures, weakness, poor appetite, vomiting, and respiratory issues	Mutations in MT-ATP6, MT-ND3, MT-CO3, and MT-ND5	Estimated 1 in 40,000 newborns	[45–47]
Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS)	Stroke-like episodes, muscle weakness and pain, seizures, headaches, vomiting, and cognitive decline	Mutations in MT-TL1, MT-ND5, MT-ND1, and others	Estimated 1 in 4,000 to 10,000 people worldwide	[48–51]
Myoclonic Epilepsy with Ragged Red Fibers (MERRF)	Myoclonic seizures, ataxia, muscle weakness, hearing loss, and dementia	Mutations in MT-TK and MT-TL1	Rare, with an estimated prevalence of less than 1 in 100,000 people	[45, 49]
Kearns-Sayre Syndrome	Progressive external ophthalmoplegia, muscle weakness, heart block, short stature, and hearing loss	Deletions in mitochondrial DNA	Rare, with an estimated prevalence of 1 in 100,000 people	[49]
Chronic Progressive External Ophthalmoplegia (CPEO)	Progressive external ophthalmoplegia, ptosis, weakness in the face and limbs, difficulty swallowing, and hearing loss	Deletions in mitochondrial DNA or mutations in MT-ND5, MT-ND6, and others	Estimated 1 in 10,000 to 50,000 people	[48, 49, 52]
Leber's Hereditary Optic Neuropathy (LHON)	Sudden vision loss in one eye, followed by the other eye weeks to months later, with little to no recovery	Mutations in MT-ND1, MT-ND4, MT-ND6, and others	Estimated 1 in 30,000 to 50,000 people	[51, 53, 54]

Table 2: Various environmental insults and the mechanisms that mediate their effects on the mitochondria leading to the development of mitochondrial dysfunction and disease.

Environmental Insults and Toxins	**Mechanisms of Effect on Mitochondria**	**Dysfunctions and Diseases**	**Relevant Publications**
Air pollution (e.g. particulate matter)	Induces oxidative stress and inflammation, leading to damage to mitochondrial DNA and proteins	Respiratory and cardiovascular diseases, cancer, Autism	[55, 56]
High-fat diet	Impairs mitochondrial biogenesis and function, leading to accumulation of dysfunctional mitochondria	Obesity, metabolic syndrome, diabetes	[34, 57–59]
Alcohol	Interferes with mitochondrial fission and fusion, leading to mitochondrial fragmentation and dysfunction	Liver disease, cardiovascular disease, neurodegenerati on	[60–62]
Pesticides (e.g. rotenone)	Inhibits complex I of the electron transport chain, leading to reduced ATP production and increased ROS production	Parkinson's disease, other neurodegenerativ	[63–68]

		e diseases, diabetes	
Heavy metals (e.g. lead, mercury)	Interferes with biogenesis, as well as increases ROS, and reduced ATP efficiency. Leading to accumulation of dysfunctional mitochondria	Neurological disorders, cancer, cardiovascular disease	[69–71]

Chapter III: Investigative Tools and Methods to Study Mitochondrial Function and Disease

Abstract

The genetic disorder Friedreich ataxia (FRDA) is a rare condition resulting from mitochondrial dysfunction. This disease leads to various symptoms, including walking, speaking, and vision difficulties. Our research indicates that the modified Idebenone conjugate (mitochondria-targeting compound-A and B (MTC-A and B)) and N-acetylcysteine (NAC) enhance ATP-dependent respiration while safeguarding against oxidative stress-related harm. These results indicate that these compounds could restore mitochondrial function in individuals who suffer from FRDA. We showed that idebenone treatment increased the transcription of genes related to mitochondrial energy metabolism. Modifications to idebenone were made to increase bioavailability and localization in the mitochondria. Functional respiration studies confirmed improved oxygen consumption rates with either molecule. Confocal microscopy showed increased mitochondrial density and Frataxin protein expression after treatment. Despite promising results, more research is required to assess the safety and efficacy of MTC-A and B in conjunction with NAC on human subjects.

Introduction

Mitochondrial dysfunction is often associated with metabolic disorders and critical illnesses brought upon by either heritable or environmental sources. As reviewed in the previous chapter, mitochondria dysfunction should be defined as an inability to fulfill the organelle's

primary purpose- to produce energy. This dysfunction can manifest in various ways, including changes to mitochondrial morphology and remodeling, decreased ATP production, and increased oxidative stress. Patients with an underlying mitochondrial disease are at particular risk for decompensation during acute conditions or increased cellular demand, resulting in multiple organ-system dysfunctions and decreased life expectancies. Research studies have developed models to accurately diagnose and treat mitochondrial dysfunction-related disorders to investigate transcriptional and metabolic patterns that reveal specific mitochondrial defects. The following experiments describe the methods of how we developed the rigorous and systematic approach in the David Brown Laboratory to analyze mitochondrial function in various physiological and pathological contexts, such as heritable diseases like Friedreich's ataxia (FRDA) and mitochondrial myopathies.

We tested the hypothesis of whether we can identify, target, and treat the specific aliment and associated mitochondrial disease. By assessing this hypothesis, we were enabled to uncover novel insights into mitochondrial function in various physiological and pathological contexts, facilitating the development of new therapeutic targets and strategies for treating mitochondrial diseases. The experiments involved a combination of in vitro studies, including biochemical metabolic assays and live cell imaging. These experiments were critical in elucidating the mechanisms of mitochondrial dysfunction in various disease states and identifying potential therapeutic targets for treating mitochondrial diseases. By utilizing the insights from previous studies in the lab, where an idebenone treatment facilitated a bypass of the mitochondrial complex I, we designed experiments to further these studies in a disease where Complex I is defective, FRDA [1–3]. Through commercial partnerships, we conjugated the idebenone molecule to become more targeted to the mitochondria and aim to reduce the reactive oxygen species (ROS)

corresponding to FRDA's inefficient mitochondria. To ensure the confidentiality of the companies involved in commercializing the discussed novel therapeutics, specific details regarding these collaborations have been omitted from this publication.

Methods

Compounds for Treatment

fforts were made to enhance the effectiveness of Idebenone (VWR International, Radnor Pennsylvania, USA), resulting in a mitochondria-targeting compound (MTC) by modifying its primary structure. The modified idebenone compounds underwent alterations such as increasing their lipophilicity and introducing positive charges, thereby boosting their ability to permeate through the mitochondrial membrane. These modifications enabled the successful synthesis and analysis of the improved idebenone compounds. Further improvements included incorporating additional functional groups like N-acetylcysteine (NAC) (Thermo Scientific, Waltham, Massachusetts, USA) to optimize their capacity for mitochondrial enhancement. Specific details regarding these modifications are not discussed herein.

Cell Lines Culture Conditions

Mouse Cell Lines

The study utilized Mus musculus myoblast C2C12 and fibroblast cell line NIH/3t3 (obtained from American Tissue Culture Collection in Manassas, VA). The media and substances used to cultivate and sustain cells were procured from Life Technologies Corporation in Carlsbad, CA. The Dulbecco's modification of Eagle medium (DMEM) was employed to maintain the cells along

with 10% (C2C12) or 15% (NIH/3t3) fetal bovine serum by volume alongside a percentage of 1% penicillin-streptomycin.

The myoblast C2C12 were differentiated into myotubes by switching to DMEM supplemented with 2% horse serum once myoblasts reached a confluency of 90-95% [4]. The differentiation process took place for 7-14 days. During the differentiation process, the culture medium was changed every other day.

Cells were maintained in a 37ºC, 5% CO2 humidified incubator and detached using a 0.25% trypsin-EDTA solution.

Human Cell Lines

Two human primary fibroblast lines, GM08399 (derived from a healthy individual) and GM03665 (derived from an FRDA patient), were procured from the Coriell Institute for Medical Research in Camden, NJ. The cells were cultured in Dulbecco's Modified Eagle Medium, containing 15% fetal bovine serum and 1% penicillin-streptomycin as supplements. Detachment of cells occurred through treatment with trypsin-EDTA solution having a concentration of 0.25%.

The non-adherent lymphoblastoid cell line (LCL) derived from B-Lymphocyte GM16243 (derived from an FRDA patient) was procured from the Coriell Institute for Medical Research in Camden, NJ. The cells were sustained in suspension using RPMI 1640 medium enriched with 10% fetal bovine serum and 1% penicillin-streptomycin. Culture conditions included maintaining the cells within a humidified incubator set at 37ºC while exposed to an atmosphere containing 5% CO2.

High-resolution respirometry

Methods were modified from our previous isolated mitochondria studies [1]. To evaluate mitochondrial activity, we employed high-resolution respirometry with simultaneous fluorometry using an Oxygraph-2k (O2k; OROBOROS INSTRUMENTS, Austria) equipped with an O2k-Fluo-Module. The measurements took place in MiR05 (a medium containing 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20mM taurine, 10mM KH_2PO_4, 20mM HEPES, pH7.1, and fatty acid-free 0.1% BSA) or Buffer Z, consisting of 105 mM K-MES, 30 mM KCl, 10 mM KH2PO4, 5 mM MgCl2, pH 7.1, and fatty acid-free 0.05% BSA. To analyze idebenone, and other mitochondrial-targeting compounds (MTC), we used mitochondrial samples at 0.5 mg/ml or cells permeabilized with 20 µg/ml (final concentration) of saponin at 3 million cells per chamber. Mitochondria samples were treated with either 1µM or 10 µM of idebenone, MTC, or vehicle control (DMSO) by incubating on ice before being added to the chamber. Cells were treated acutely or incubated with 1µM or 10 µM of idebenone, MTC, or vehicle control (DMSO) for 24 hours before being loaded and permeabilized in the chamber. For extended respiration studies, substrate injections consisted of succinate (10mM), H2O2 (0.1µM), ADP (5mM), pyruvate (5mM), malate (2mM), carboxyatractyloside (Catr) at a concentration of 5 µM and an uncoupler (U) CCCP at a final concentration volume of 0.5 µM were utilized. All tests were conducted at a temperature of 37 °C.

Extracellular Metabolic Flux Analysis

Non-differentiated Adherent Cells

Oxygen consumption rate (OCR) was measured using an XF96 Seahorse Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, California, USA). Cultured C2C12, GM08399, or

GM03665 cells were seeded at a density of 1.5 x 104 per well in their respective supplemented media on a Seahorse XF96 Cell Culture Microplate. Cells were then incubated overnight at 37ºC in 5% CO2 for adherence. Following adherence, cells were pretreated for 4 or 24 hours with idebenone, NAC MTC, or vehicle control, depending on the assay requirements. After pretreatment, cells were injured by adding 500uM H2O2 and incubated for an additional 4 hours. The cells were washed twice with supplemented XF media (XF base media plus 1mM pyruvate, 2mM glutamine, and 10mM glucose) before adding a final volume of 180uL per well. An XF Cell Mitochondrial Stress Test assessed the bioenergetic status of the cells through injection of ATPase inhibitor oligomycin (1ug/mL), inner membrane uncoupler FCCP (2uM), and complex III inhibitor antimycin A (2uM). Data are expressed as pmol of O2 per minute per 1.5 x 10^4 cells.

Differentiated Cells

The previously discussed differentiation protocol was performed within each well of the XF96 Seahorse plate. Cells were seeded at 200 cells per well at an initial density and incubated for 24 hours to ensure adherence. Culture media was then changed to differentiation media (DMEM supplemented with 2% Horse Serum and 1% Penicillin-Streptomycin antibiotic). Cells were incubated for six additional days with media changes every other day. Cells were then dosed with idebenone, NAC MTC, or vehicle control for 4 or 24 hours before a subsequent 4-hour incubation with a 1mM H2O2 insult. The XF Cell Mitochondrial Stress Test described above was performed to measure OCR.

Suspension cultured Cells

The Non-adherent Lymphoblastoid patient Cell Line, GM16243, was bioenergetically assessed using a novel approach by pretreating the cells in their designated supplemented media for 24 hours with idebenone, NAC MTC, or vehicle control. Cells were then spun down at 200xg for 5 minutes and resuspended in fresh supplemented XF media to a final concentration yielding 1.25 x 10^5 cells per 50uL for each well in the Seahorse XF96 Cell Culture Microplate. Before seeding the plate, the XF96 Cell Culture Microplate was coated with 67.2ug/mL of Cell-Tak (BD Biosciences) at 10uL per well, then activated using 100mM Sodium Bicarbonate (pH 8.5) with 20uL per well and incubated at room temperature for 30 minutes. Following activation, cells were washed twice with 200 uL of sterile water. 50uL of the treated cells were then added to each well. The plate was spun at 200xg for 1 minute with no breaking to ensure even distribution of the cell suspension. Due to the severity of the patient, no H2O2 insult was performed. The bioenergetic status of the cells was assessed using an XF Cell Mito Stress Test as previously described.

Mitochondrial Biogenesis and Endogenous Antioxidant Gene Quantification with qPCR

The Applied Biosystems ViiA 7 Real-Time PCR system assessed the relative expression levels of PGC-1α and NRF2. C2C12 cells were cultured for three days before exposure to idebenone, NAC MTC, or vehicle control, followed by an additional 24-hour incubation period. RNA extraction utilized RNeasy Mini Kit with DNAse I treatment according to the manufacturer's instructions (Qiagen, Valencia, CA). The TaqMan Universal PCR Master Mix was combined with the ABI PRISM 7900 Sequence Detection System instrument for qRT-PCR assay. Pre-designed primers were used for PGC-1α, NRF2, and β -actin, along with prevalidated FAM-labeled TaqMan probes from Invitrogen. Target gene expression was normalized in cell cultures by measuring β -actin

RNA levels simultaneously while the derivation of $2^{-\Delta\Delta CT}$ values determined relative mRNA fold change.

Statistical Analysis

GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA) was utilized for statistical analysis. One-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparisons test for posthoc analysis to determine statistical significance at a p-value of less than 0.05. The data is presented as mean ± standard error of the mean (SEM).

Results

Using the Applied Biosystems ViiA 7 qRT-PCR system, we saw a significant increase expression of biogenesis marker PGC-1α ($p < 0.05$) and a trending increase in endogenous antioxidant marker NRF2 after a 24-hour incubation in the idebenone (see Figure 1). With this increased expression of these markers, we wanted to assess the functional effects of these increased markers through respiration studies, specifically in cells with deficits in mitochondrial function.

The Oxygraph-2k was used to measure the respiratory function of permeabilized LCL GM16243 cells isolated from an FRDA patient. As shown in Figure 2, we saw a minimal increase in respiration following the complex I specific substrates with Glutamate and Malate, indicating a deficiency in this complex due to the associated effects of FRDA. MTC-A showed a moderate increase (p-value 0.2906) in Complex II respiration following succinate-induced stimulation. The overall respiration rates were noticeably low compared to previous studies in permeabilized cells. The comparatively low respiration rates indicated possible damage to the cells in permeabilization.

The XF96 Seahorse Extracellular Flux Analyzer was used with intact, non-permeabilized cells to minimize cellular damage and accurately evaluate mitochondrial function due to their low respiration rates. With the MTC-C inducing further insult to the cells in the O2k studies, only MTC-A and B were further assessed in the FRDA patient LCL GM16243. As highlighted in Figure 3, MTC-A and B showed moderate improvements in maximal respiration at the low concentration of 1uM (p-value 0.1665 and 0.2393, respectively), with higher concentrations inducing further insult or cytotoxicity. Figure 4 shows the ATP-Dependent respiration states. Both compounds at 1uM significantly improve respiration ($P < 0.05$) compared to the vehicle control, DMSO, whereas the higher concentrations resulted in a cytotoxic event.

To add further evidence of the efficacy of MTC-A and B, we conducted additional experiments using healthy patient fibroblast GM08399 and FDA patient fibroblast GM03665. These studies used a concentration range of 0.25 uM to 1uM MTC-A and B to evaluate their effect on maximal and ATP-dependent respiration in intact cells. The results obtained from these experiments (shown in Figures 5 and 6) demonstrated that MTC-B could significantly improve ($p < 0.05$) maximal and ATP-Dependent respiration compared to vehicle control (DMSO) and positive control (idebenone) in GM03665 (FRDA) patient fibroblasts, with the most effective concentration being 1uM.

To examine these compounds' protective effects and mitochondrially targeting mechanisms on more complex tissues, we differentiated C2C12 mouse myoblast into fibers in XF96 well plates before treatments. Following differentiation, the C2C12 myotubes were treated with MTC-A and B at 1uM and 3 uM concentrations for 4 and 24 hours before a 1mM H2O2 insult. The results (shown in Figure 7) demonstrated that MTC-A and B improved maximal respiration at 1 uM for 4 and 3 uM for 24 hours.

Further modification of these compounds improved their efficacy and specificity for targeting the underlying molecular deficiencies in metabolic-related diseases by conjugating them with N-acetylcysteine (NAC), a potent antioxidant. These modifications were accompanied by significant improvements in maximal compared to vehicle control, DMSO, and idebenone-positive control. Differentiated cells were treated with concentrations of 1um and 10 uM. Figure 8 indicates that cells treated with 1 and 10uM produced significantly better Maximal respiration than the injured vehicle control ($p < 0.05$).

Discussion

Following our previous work in idebenone, we saw mitochondrial restoration in the presence of ROS [1]. The results from this study indicated the effects of idebenone on enhancing mitochondrial function, as evidenced by a significant increase in respiratory control ratio (RCR) and improvement in mitochondrial membrane potential ($\Delta\Psi m$) following an insult to the mitochondria. The qRT-PCR studies in Figure 1 supported these data, where PGC-1α and NRF2 showed increased expression.

Notably, in the lab's previous study, there was evidence of a complex I bypass after rotenone injection, which acts as a complex I inhibitor [1]. This bypass effect facilitated the idea that utilizing idebenone and idebenone-like MTC would improve a metabolic disorder, such as FRDA, where complex I deficiencies are seen. This aspect of FRDA is seen in the respiration data in the Oxygraph-2k in Figure 2, where the glutamate malate had a minimal increase in respiration, suggesting that the FRDA patient's cells exhibited a deficiency in complex I, as well as low rates of respiration which is consistent with the known effects of this disease [3]. Due to the permeability

protocol potentially inducing further injury, studies using the XF96 Seahorse Extracellular Flux Analyzer became essential. The results obtained from this instrument indicated recoverability in mitochondrial function, whereas the Oxygraph-2k did not.

To yield a more mitochondrially targeting molecule, modifications to idebenone were alterations to the molecule's cationic and lipophilic properties to localize to the negatively charged, fatty acid-composed organelle [5]. While reviewing these studies, the molarity differences between these modified-idebenone MTCs and the positive controls should be accounted for. While the positive controls may be used to compare the MTCs' functionality, the integrated molecule's working concentration (idebenone and/or NAC) is substantially less than that of the positive control. This could indicate that the bioavailability of the molecule has increased due to the decreased functional concentration. This is a rather notable benefit when considering the value of these modifications. Idebenone has been seen to be toxic at higher concentrations, thus negating any benefits a patient with FRDA may see to treat their Complex I deficiency [6]. This toxicity can be seen in Figures 3 and 4, where the higher concentrations of the MTC-A and B, 3 uM and 10 uM, reduced maximal and ATP-dependent compared to the untreated vehicle control. In the subsequent studies where healthy and FRDA patient cells were used, lower concentrations became the focus to further understand the pharmacokinetic properties of the modifications made to idebenone. These studies indicated improved responses and targeting mechanisms due to the modifications made to idebenone. Notably, in Figure 5, when comparing the lowest concentration, 250 nM of MTC-A and B, maximal respiration rates were comparable to the positive control of 1 uM Idebenone. The following figure, Figure 6, indicated further improvements at the lowest concentration in both MTC-A and B, with the ATP-dependent respiration being higher than idebenone with MTC-A and significantly higher than Idebenone ($\#p < 0.05$).

By utilizing differentiated C2C12 cells, we further explored the targeting and bioavailability aspects of the molecules on more developed, robust tissues. These cells were exogenously damaged with hydrogen peroxide to mimic an insult or disease state and to identify the antioxidant properties of the MTCs. Further modifications by incorporating the potent antioxidant, N-acetylcysteine (NAC), into the molecules was merited due to the mixed results from the treatments, both in the MTCs and the positive Idebenone controls denoted briefly in Figure 7. The results in the following study, shown in Figure 8, exhibited dramatic improvements with the additional conjugations made. Subsequent studies on differentiated FRDA fibroblast should be performed to indicate clinical translatability further. The limitations on uniform cell viability through the differentiation process within each well should be accounted for. Protein quantification by a bicinchoninic acid (BCA) assay or nuclear fluorescence staining could normalize the total tissue within each well [7, 8].

Subsequent studies using confocal microscopy indicated an increase in mitochondrial density and an increase in the frataxin protein following treatments of the MTC. These studies were done using the same healthy patient fibroblast, GM08399, and FDA patient fibroblast, GM03665, which were both transfected with a Frataxin localizing GFP plasmid, as well as counter-stained with MitoTracker Deep Red and treated with MTCs. Images were taken following treatment and then shared with commercial partners for analysis. Due to the proprietary nature of these images and protocols, no data can be shared. Still, results can be inferred using the improved response in both the PCR studies and the functional respiration results. Comparative fluorescence images are shared in Figure 9, wherein in the untreated state, the FRDA patient has a significant deficit in fluorescent intensity in Frataxin protein and a trending deficiency in mitochondria intensity. These baseline images indicate that the morphology of the FRDA cells in an untreated

state serves as an injury model to suggest improvements following treatment by monitoring the mitochondrial network and frataxin content.

Conclusions

In summary, the results of this study provide preclinical evidence that MTC-A and B, in conjunction with NAC, could have potential therapeutic effects for patients suffering from Friedrich's ataxia and other mitochondrial diseases. These compounds can potentially restore mitochondrial function in these patients by improving their maximal and ATP-dependent respiration and protecting them against further oxidative damage. These studies provide a preclinical foundation for further research to evaluate the safety and efficacy of these compounds in humans. Collaboration and additional development will further advance these molecules' delivery and pharmacokinetic properties, thus improving the lives of those suffering from FRDA and other mitochondrial diseases.

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Figures

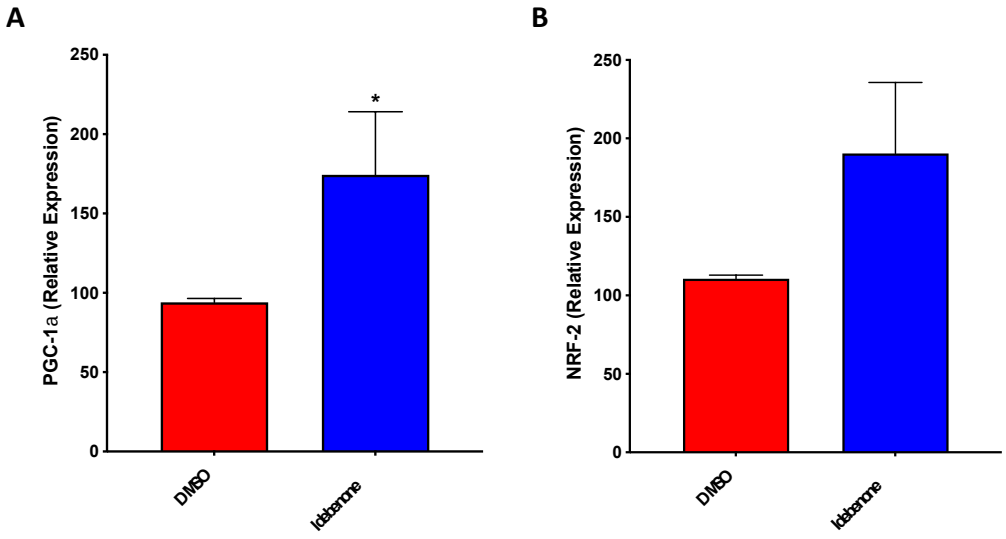

Figure 1: Idebenone increased expression of PGC-1α in myoblasts. C2C12 Myoblasts were treated for 24 hours with Idebenone or vehicle control (DMSO) before RNA extraction and measurement of PGC-1α and NRF2 using qRT-PCR. A) Idebenone significantly increased PGC-1α expression (* $p < 0.05$). B) Idebenone marginally increased NRF2 expression ($p = 0.1339$).

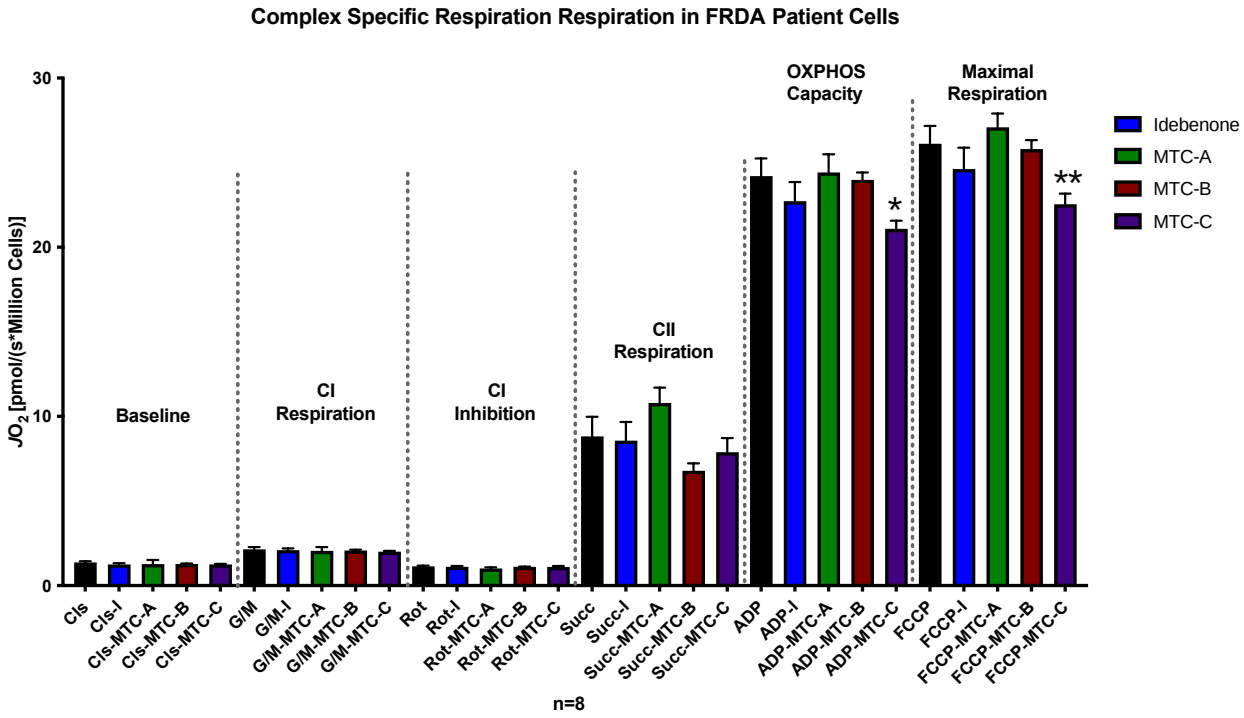

Figure 2: Complex I deficiencies in the Electron Transport Chain and low Mitochondrial respiration rates in Friedreich's ataxia (FRDA) patient cells. Lymphoblastoid Cells (LCL) from FRDA patient B-Lymphocyte (Coriell Institute cell reference number: GM16243) were exposed to a 24-hour treatment of MTCs before permeabilization and assessment of complex specific respiration using the Oxygraph-2k. Rates of respiration are notably low in complex I, as well as other states of respiration, indicating the severity of the disease and possibly increased sensitivity to permeabilization.

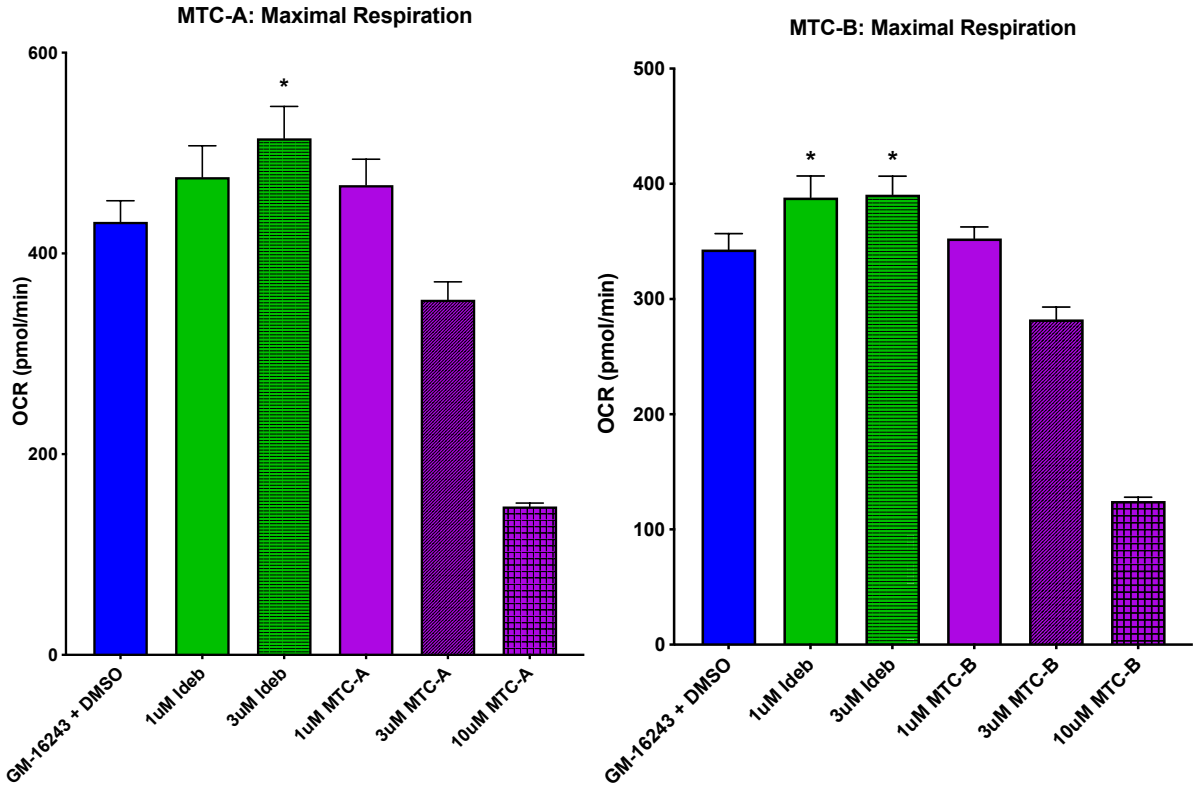

Figure 3: MTC-A and B did not significantly improve Maximal respiration in Friedreich's ataxia (FRDA) patient Lymphoblastoid Cells (LCL).

Maximal Respiration Data in FDA patient LCL GM16243, after 24-hour treatment of MTC-A and B, data indicated no significant improvement in maximal respiration. Idebenone showed significant improvement in respiration (* $p < 0.05$) at 3 uM in both studies and 1 uM in the MTC-B study compared to the untreated vehicle control (DMSO). Low rates of respiration at 3uM and 10 uM indicate toxicity.

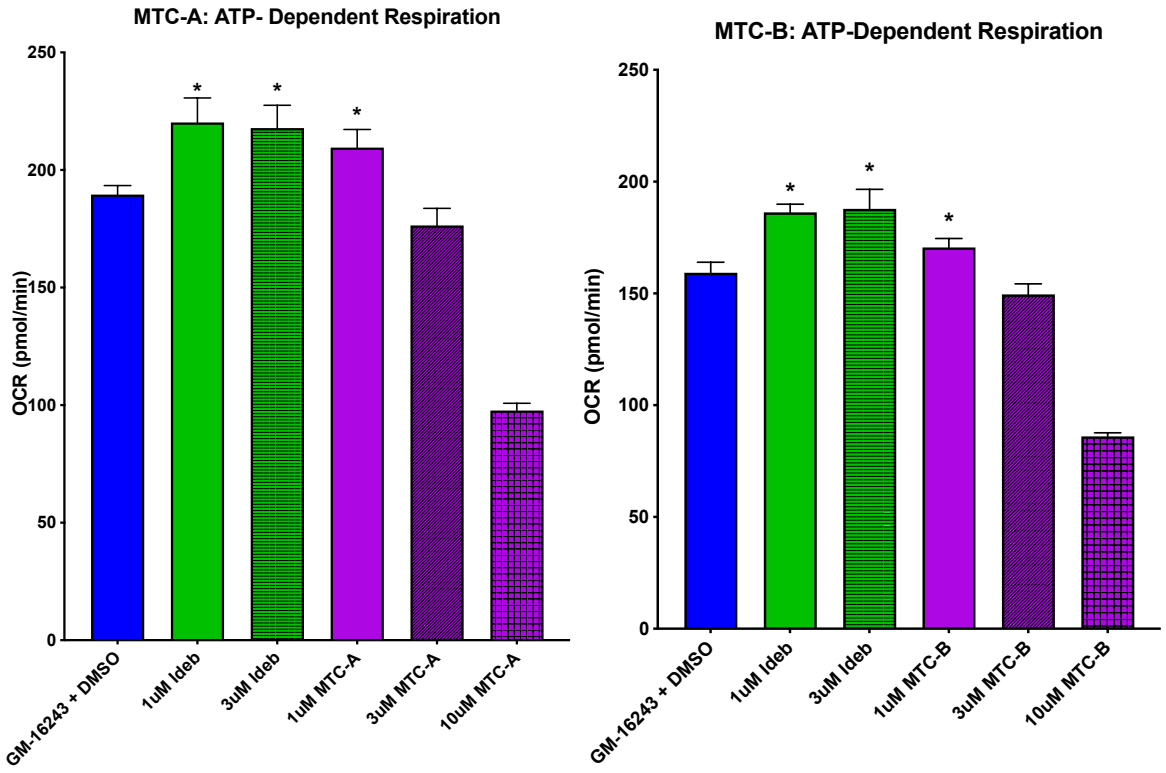

Figure 4: MTC-A and B improve ATP-Dependent Respiration in FRDA LCL.

ATP-Dependent Respiration Data in FDA patient LCL GM16243, after 24-hour treatment of MTC-A and B. Data indicated significant improvement (*$p < 0.05$) in respiration in both 1 uM and 3uM concentrations of Idebenone and 1 uM of both MTC-A and B compared to the vehicle control (DMSO) treated FRDA patient cells. Low rates of respiration at 3uM and 10 uM indicate toxicity.

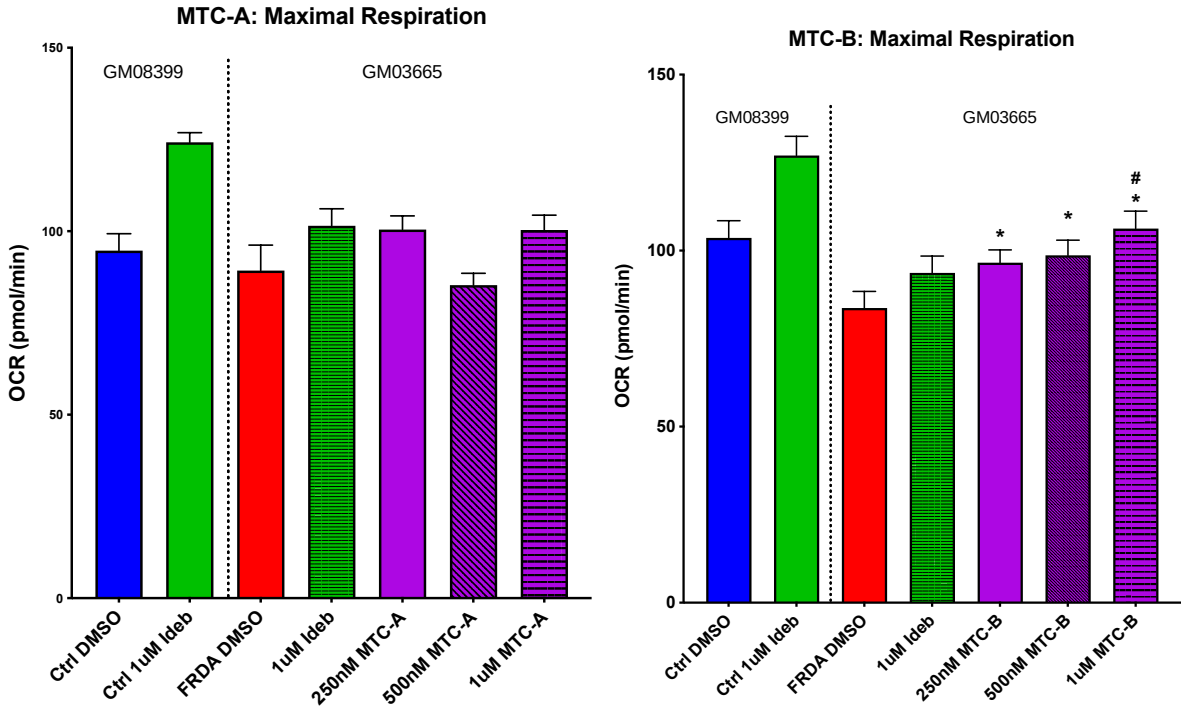

Figure 5: MTC-B improves Maximal respiration in FRDA fibroblast.

Maximal Respiration Data in healthy patient fibroblast (Coriell Institute cell reference number: GM0839) and FDA patient fibroblast (Coriell Institute cell reference number: GM03665) after 24-hour treatment of MTC-A and B at multiple concentrations (250 nM, 500 nM, and 1uM). MTC-B had significant improvement (*p< 0.05) respiration with all concentrations compared to the vehicle control (DMSO) treated FRDA patient cells, with 1 uM having even further significance (#p< 0.05) when compared to 1 uM Idebenone. Comparable respiration rates at the lowest concentration, 250 nM of the MTCs compared to 1 uM Idebenone treatment, indicate improved bioavailability and targeting mechanisms due to modifications.

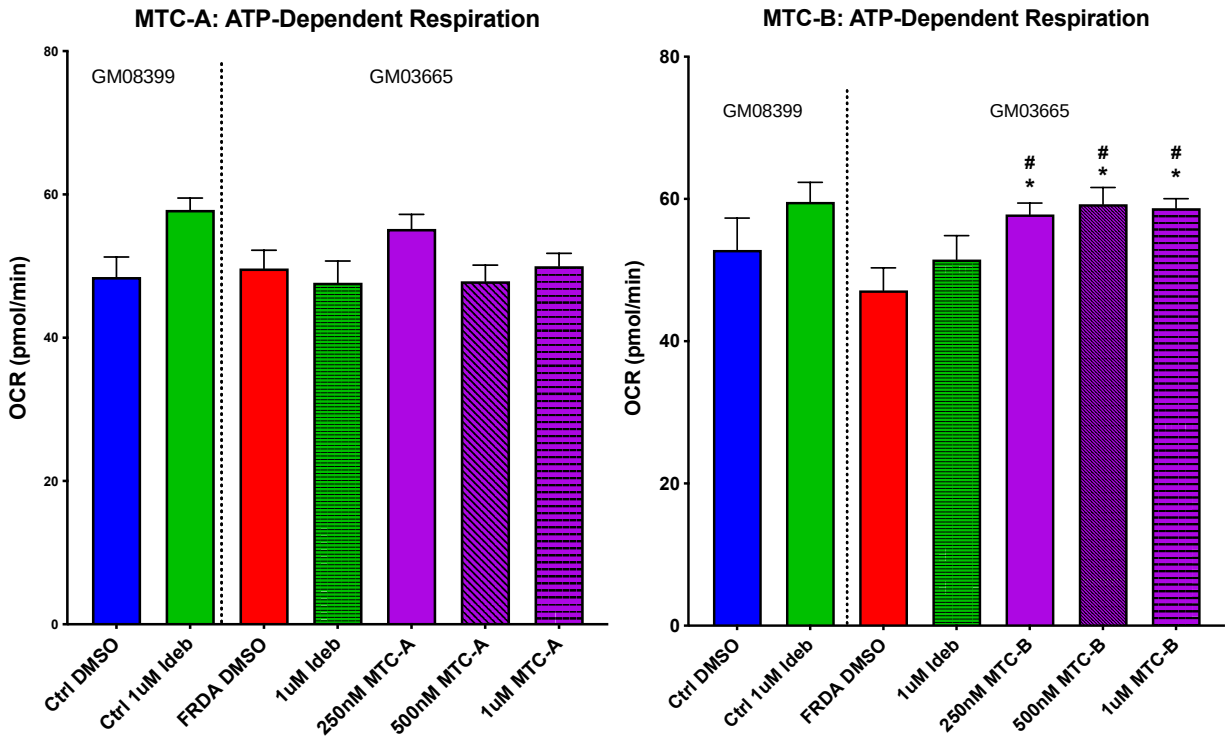

Figure 6: MTC-B improves ATP-Dependent respiration in FRDA fibroblast.

ATP-Dependent Respiration Data in healthy patient fibroblast (GM08399) and FDA patient fibroblast (GM03665), after 24-hour treatment of MTC-A and B with multiple concentrations (250 nM, 500 nM, and 1uM). MTC-B had significant improvement (*$p < 0.05$) respiration with all concentrations compared to the vehicle control (DMSO) treated FRDA patient cells and the FRDA patient cells treated with idebenone (#$p < 0.05$). Improved respiration rates at the lowest concentration, 250 nM of MTC-A and all concentrations of MTC-B, compared to 1 uM Idebenone treatment, indicate enhanced bioavailability and targeting mechanisms due to modifications.

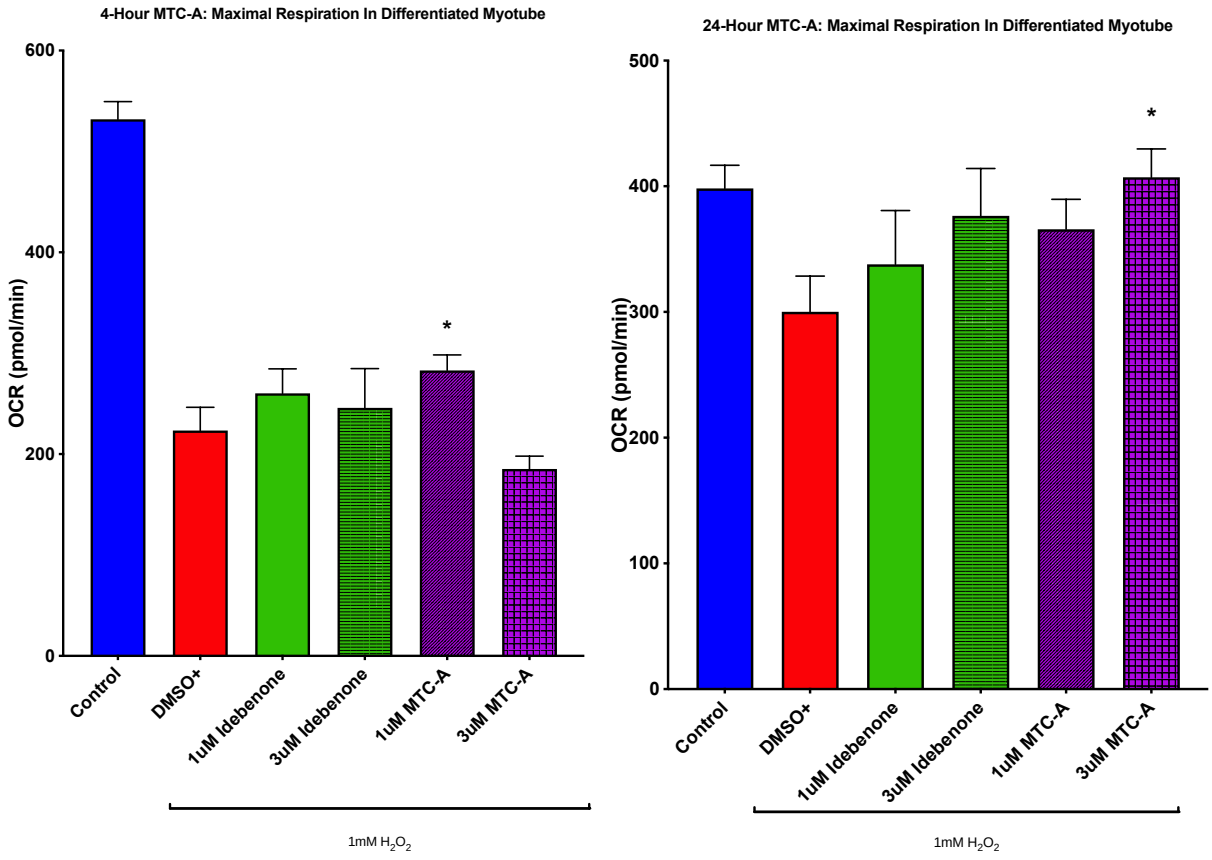

Figure 7: MTC-A improves Maximal respiration in injured C2C12 Myotubes.

Maximal Respiration data in Differentiated C2C12 Myotube after exposure to both 4 and 24-hour treatment of MTC-A. Data indicates that MTC-A significantly improves (*$p < 0.05$) respiration with 1uM at 4 hours and 3uM at 24 hours of treatment, compared to the injured vehicle control (DMSO+).

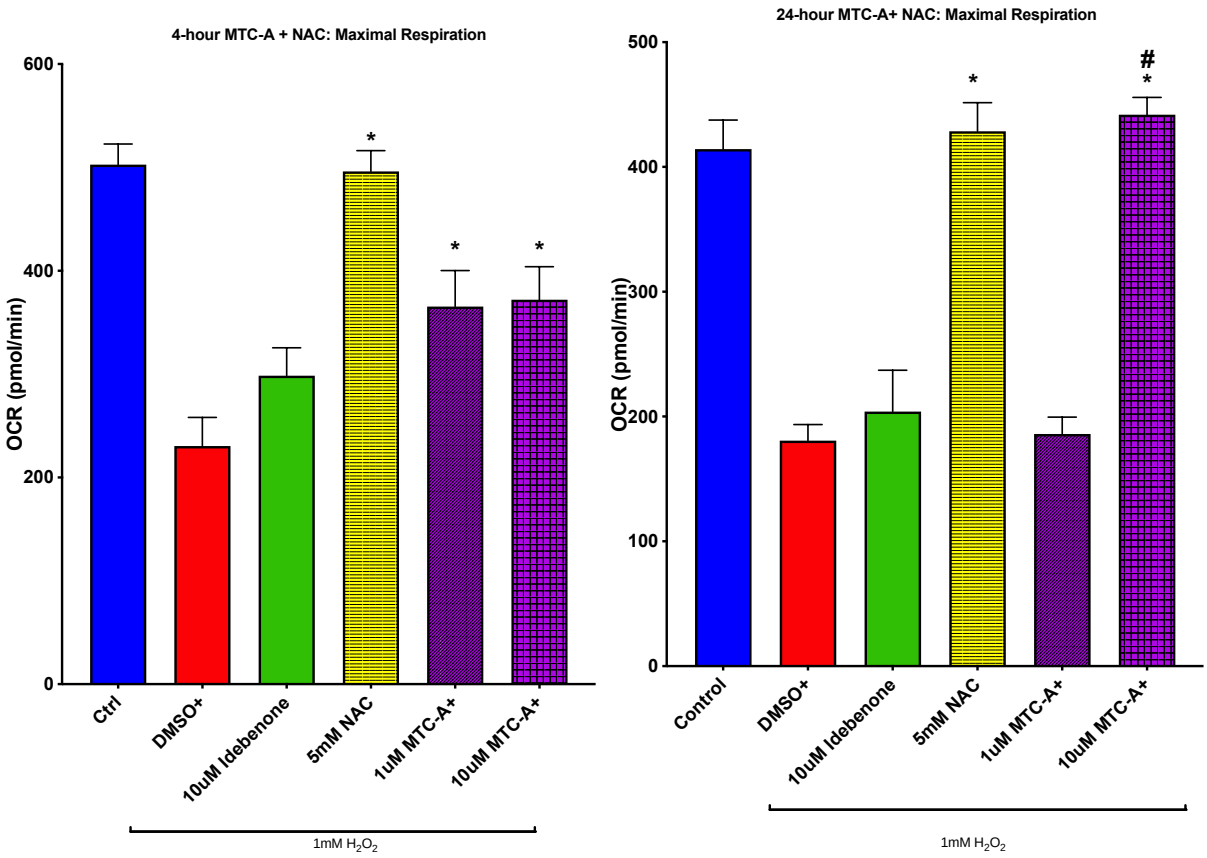

Figure 8: MTC-A conjugated with N-acetylcysteine (NAC) protects cells from oxidative damage and further improves Maximal respiration in injured C2C12 Myotubes.

Modifications to the MTC-A by incorporating NAC yielded more significate improvements in respiration and protection in both 4- and 24-hour treatments. Data indicates that MTC-A significantly improves (*$p < 0.05$) respiration with 1uM and 10uM at 4 hours of treatment, compared to the injured vehicle control. Additionally, there were significant improvements in respiration at 10uM at 24 hours of treatment, compared to the injured vehicle control (DMSO+) (*$p < 0.05$) and idebenone (#$p < 0.05$), indicating a more mitochondrially protective molecule than idebenone alone.

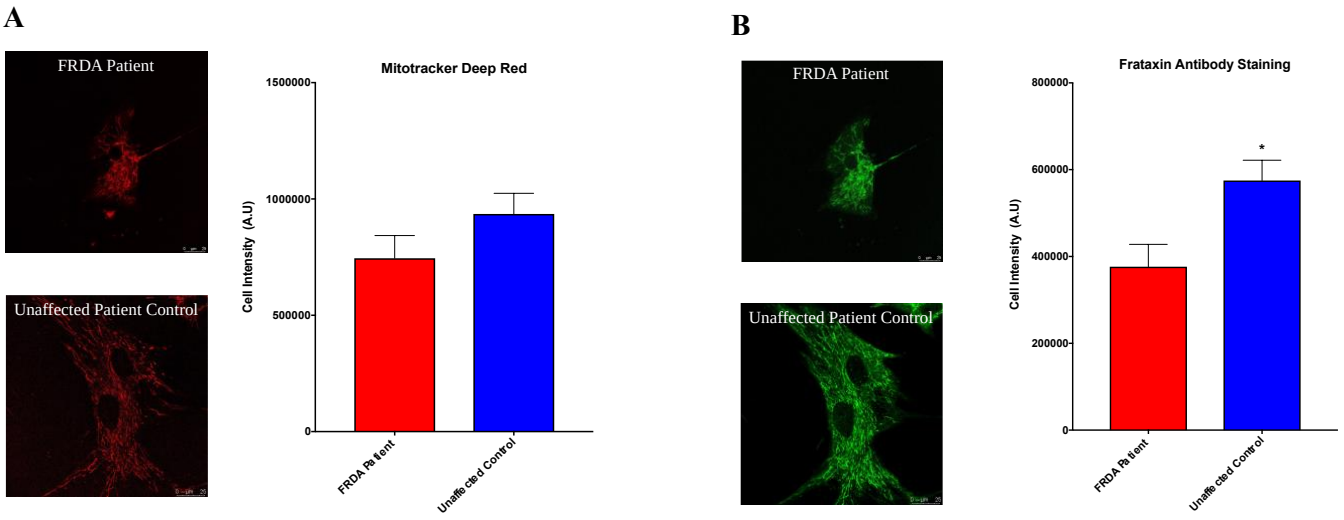

Figure 9: Variations in FRDA Patient and Healthy Patient Cells fluorescence Insitity with Mitotracker Deep Red and GFP FXN plasmid with Frataxin antibody Staining.

A) FRDA patient cells were used to assess the effects of MTCs on mitochondrial morphology and visual density. B) Additional studies aimed at assessing the increase in frataxin protein following MTC treatment by comparing FRDA patient cells to healthy patient cells transfected with a GFP FXN plasmid and Frataxin antibody Staining. Critical differences in mitochondrial morphology, network, and frataxin content were recognized. Untreated Baseline images are shown for context.

Chapter IV: Environmental Impact on Mitochondrial Development and Functionality

Abstract

Mitochondrial health is critical for normal brain development, and mitochondrial toxicants impair energy production and induce mitophagy, resulting in network fission. This can substantially impact cellular and molecular pathways essential for brain development. Recent studies have shown that triclosan, a once widely used antibacterial and antifungal agent, acts as a mitochondrial uncoupler. However, the impact of triclosan on mitochondrial function in the developing brain has yet to be thoroughly investigated. We performed a series of experiments designed to assess the effects of triclosan on mitochondrial form and function in the developing *Xenopus laevis* tadpole brain. We injected tadpole brains with TMRM, a fluorescent reporter of mitochondrial membrane potential. After two hours, we imaged the brain using high-resolution confocal microscopy to obtain a baseline level of TMRM fluorescence, then immediately treated tadpoles with either triclosan (10uM, 5uM, 1uM, 0.5uM 0.1uM), FCCP (0.5uM), or control, and then continued imaging the brain once every five min for 30 min. We found that 10uM triclosan significantly decreased TMRM fluorescence in mitochondria in the end feet of radial glial cells in ~ 15-20 min; lower concentrations operated over a longer time course. We also observed that mitochondrial networking was disrupted as they underwent fission. In addition, we measured metabolic function in vivo in whole tadpoles using an XF Seahorse Flux Analyzer, acutely exposing embryos to either triclosan (30uM, 10uM, 1uM) or control. Triclosan immediately increased oxygen consumption rates dose-dependently, suggesting mitochondrial uncoupling. Last, we evaluated additional in vivo fluorescent reporters to assess triclosan's effects on calcium, pH, and ATP levels. The results show that triclosan impairs mitochondrial form and function in developing neural tissue.

Introduction

Triclosan (TCS) was first introduced in 1970 as an antimicrobial additive; where it eventually entered thousands of health and home care products, including deodorants, mouthwash, toothpaste, cosmetics, shampoo, skin creams, plastic kitchenware, children's products, shoes, and textiles. Companies marketed TCS with claims of prolonged shelf life and increased cleanliness [1]. Yet TCS has been implicated in many adverse health outcomes, including impaired metabolism and even some aspects of cognitive function [2]. After an FDA risk assessment, TCS was banned from commercial soaps [3]. It is still widely prevalent in other goods and products, with no explicit medical claim on the product's label [4]. Before the FDA decision, TCS was among the top ten most commonly detected organic wastewater compounds in the United States [5–8] and is readily absorbed through the skin [9]. TCS is observable in human urine, breastmilk, blood, and newborn's umbilical cord at astonishing rates [10–16]. Children born to mothers who had detectable levels of TCS in their urine at the child's birth have been associated with a decrease in IQ score, verbal comprehension, and perceptual reasoning when tested at eight years-of-age [17]. This implies that TCS may impair brain development, and a study in rats showed that there were autism-like social deficits and repetitive symptoms akin to obsessive-compulsive behavior in the adult offspring exposed to TCS during pregnancy [18]. Some have suggested that the increased exposure to TCS in the last 20+ years may explain the growing number of autism spectrum disorder (ASD) cases. Autism rates rose from 1/149 children in 2000 to about 1/40 children in 2016 [19], with the mean age of first birth only changing by roughly 2.5 years in that same time [20]. Epidemiological data suggest that an environmental component likely explains, at least in part, some of the increase in the incidence of autism.

Only a handful of studies have examined the effects of TCS exposure on the brain. In the snail nervous system, TCS significantly decreases action potential amplitude. Where in mouse neuromuscular end junctions, TCS increases spontaneous vesicle release and reduces membrane potential [21]. Additionally, decreased synaptic density and increased apoptosis are seen in the brains of developing zebrafish after a TCS treatment [22]. To fill in some of the gaps in our understanding of how TCS affects the brain, we conducted several experiments to examine the effects of TCS on mitochondrial function in *Xenopus laevis* tadpoles, including investigations to determine how TCS affects neural progenitor cells (NPCs, also known as radial glial cells), see Figure 1 for reference. These cells divide and give rise to neurons, and they may be especially susceptible to TCS because neuronal proliferation is controlled by mitochondrial activity within this population of cells [23]. A growing body of evidence indicates a long-term, detrimental effect on the mitochondria due to TCS's lipophilic properties directly targeting the phospholipid membrane. TCS disrupts mitochondrial form and function through its uncoupling properties [24], which we hypothesize as its primary influence on the disruption of the proton gradient. At this point, there are no studies that explore TCS's effects on mitochondrial changes to NPCs, and few studies examining the effects on overall physiological changes *in vivo.*

To further investigate the toxicological effects of TCS in the developing brain *in vivo,* we used *Xenopus laevis* tadpoles (also known as the African clawed frog). Tadpoles are an ideal animal model because we can tightly control environmental concentrations of TCS as well as monitor morphological changes as the brain rapidly develops. Additionally, due to the tadpole's transparent skin over the brain, we can observe the real-time response to TCS in individual cells and even in specific mitochondria. This makes it an ideal model to test the hypothesis of TCS impairing mitochondrial function in developing tissues. Additional investigations into the

mechanism of action of TCS are essential to uncovering ways of ensuring no further damage to future populations and aiding in possible approaches to reversing potential impairments to mitochondrial function.

Methods:

Chemicals and Reagents:

TCS (Irgasan, CAS 3380-34-5, 97.0%),

FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, CAS 370-86-5, 98%)

Oligomycin

Antimycin A

MS222 (Tricaine methanesulfonate, CAS 886-86-2, 98%)

was purchased from Sigma-Aldrich (St. Louis, Mo, USA).

-60x E3 Media: 297.7413mM NaCL, 10.7309mM KCL, 19.7265mM CaCl2H4O2, 24.0531mM Cl2H12MgO6, added to Millipore H2O. Using HCL, pH to 7.4.

-0.01% MS-222, 1x E3 Media: 50mL 0.2% MS-222, 16.5 mL of 60× stock E3 medium, brought to 1 liter using Millipore H2O.

Animal husbandry:

Xenopus laevis were maintained through established methods [25]. Adult frogs were raised at a temperature of 18°C and maintained with standard sinking frog food given three times per week. Breeding was induced between two healthy adult frogs with subcutaneous injections of human chorionic gonadotropin. This would yield a clutch of roughly 2000 fertilized eggs overnight. Adult frogs were removed, and embryos were raised to either stage 22-25 (3-4 days old) or tadpoles to stage 46-47 (7-10 days old).

Mortality Analysis:

Treatment groups were established to assess triclosan's effects on the overall survivability and range of concentration on the *Xenopus laevis* tadpoles. Embryos in stage 25 were placed in treatment groups of either vehicle control, 0.5uM FCCP, or a TCS concentration of 0.1uM, 0.5uM, 1uM, 5uM, 10uM, or 50uM. A population of 12 embryos a bowl, at two bowls a treatment, repeated in separate clutches (n=48). All treatments were diluted in Steinberg's solution. Survival rates were measured over 14 days and classified based on active heartbeat under a brightfield microscope. Tadpoles with no heartbeat were considered to be deceased.

Mitochondrial labeling:

Whole Embryo Imaging

Stage 25 embryos were incubated in 10 uM TMRM diluted in Steinburg's solution on a rocker for 2 hours. Embryos were removed from the TMRM solution and washed with fresh Steinberg's solution twice for 15 minutes to remove any access stain before imaging.

Brain Imaging

Stage 46 tadpoles were anesthetized using 0.01% MS-222. Mitochondria in the brain were then labeled via intraventricular injection through a fine-tip glass micropipette filled with 12.5 uM TMRM diluted in DMSO and mounted on a pico-spritzer set to 32 psi of pressure and 8 milliseconds. Animals were then incubated for 24 hours before imaging.

Triclosan Treatment for Imagining:

Embryos or tadpoles were placed in TCS (30uM, 10uM, 5uM, 1uM) or positive control FCCP (0.5uM) made in 0.01% MS-222. All experiments shown are acute exposures to TCS. Images were taken every 5 minutes following acute TCS treatment. Images were normalized based on pre-treatment fluorescence intensity.

Confocal imaging and analysis:

Embryos and tadpoles were imaged using a Lecia SP8 confocal microscope using standard confocal settings. Imaging parameters were set to repeat imaging settings, where intensity, speed, resolution, gain, position, and z-plane were selected, and set based on pre-treatment parameters and maintained throughout the experiment. Images were analyzed using ImageJ, where the intensity of z-plane projections was assessed over time.

Extracellular Metabolic Flux Analysis:

Oxygen consumption rate (OCR) was measured using an XFe24 Seahorse Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, California, USA). The day before the planned assay, the XFe24 Extracellular Flux Assay Kit cartage was hydrated using 1mL of XF Calibrant for every

24 wells, then placed into a 25ºC incubator. Healthy eggs were incubated at 25ºC following fertilization. Embryos were incubated until stage 23, roughly 48 hours post-fertilization. Using the hydrated cartridge, either vehicle control, 300uM, 100uM, or 10uM TCS was loaded into well A at a volume of 70uL for the final concentrations of 30uM, 10uM, or 1uM per well. An XF Cell Mitochondrial Stress Test was prepared to assess the bioenergetic status of the embryos by injecting ATPase inhibitor oligomycin (10uM) in well B, inner membrane uncoupler FCCP (2.5uM) in well C. Complex III inhibitor, antimycin A (1uM) with complex I inhibitor rotenone (1uM) in well D. 22 embryos were loaded into Agilent Seahorse Islet Capture Microplates, with provided micro screens encasing each embryo. Embryos were washed twice with 0.01% MS-222, 1x E3 media before adding the final volume of 630uL to account for increasing volume changes from each injection. An XF Cell Mitochondrial Stress Test was carried out with the heating element turned off on the XFe24 Seahorse Extracellular Flux Analyzer before assay. Data are expressed as percent control of pmol of O_2 per minute per each well's stabilized baseline measurement.

Statistical analysis:

Two-way ANOVAs and subsequent graphs were done using Graph Pad Prism. These were used to analyze the impact of the two factors (time and dose) on the response variables (e.g., change in TMRM intensity).

Results

Experimental groups were established to assess the degree of mortality of triclosan (TCS) and dosage levels on *Xenopus laevis* tadpoles. The survival rates of the tadpoles were monitored at regular intervals over 14 days after exposure to varying concentrations of TCS and the positive control, 0.5 um FCCP. The results, depicted in Figure 2, indicate that concentrations exceeding 5um TCS were substantially toxic, leading to 100% fatality within hours for all tested tadpoles. Although FCCP was also found to be lethal, it exhibited delayed toxicity compared with high doses of TCS, which was valuable information for future experiments. While tolerable concentration levels could extend as low as 1 uM for prolonged durations, mortality rates surged drastically during the last day using this dose range. This suggests that caution should be taken when considering use cases with such levels or durations beyond seven days. Interestingly, no significant differences in survivability were observed between control groups and TCS dosages ranging from 0.1 uM to 0.5uM.

Confocal microscopy was employed on a Leica SP8 confocal microscope to assess the effects of acute treatment on stage-25 *Xenopus laevis* embryos prior to mortality in the high concentrations used above. Embryos were stained with TMRM, a membrane potential dye, to evaluate the mitochondrial activity and membrane potential. For normalization purposes, initial pretreatment images were captured before treating the tadpoles with TCS for 30 minutes while capturing images every 5 minutes. The results from confocal microscopy analysis showed a significant decrease in mitochondrial activity and membrane potential in tadpoles exposed to TCS compared to the control (see Figure 3 for reference). Where animals treated with 10 uM had roughly a 60% reduction in fluorescence intensity 30 minutes after acute exposure.

Further studies were conducted to investigate the activity of mitochondria in stage-25 *Xenopus laevis* embryos. The XFe24 Seahorse Extracellular Flux Analyzer measured the oxygen consumption rate (OCR) in live embryos in the XF Islet Plate. Initial OCR measurements were made before embryos were exposed to varying concentrations (30uM, 10uM, or 1uM) of TCS and control solutions. As shown in Figure 4, after exposure to an ATPase inhibitor, oligomycin (10uM), and inner membrane uncoupler FCCP (2.5 uM), OCR levels increased overall for those tadpoles exposed to 30uM TCS even after undergoing oligomycin treatment. This suggests that TCS acts as a mitochondrial uncoupler by inducing loss of ATP-dependent respiration ($p < 0.001$).

To better understand the mechanisms behind TCS-induced mitochondrial dysfunction, we conducted additional confocal imaging experiments using stage-47 tadpoles to examine its impact on developing brains. Intraventricular injections of TMRM were administered to the tadpoles. Following a 24-hour incubation period, tadpoles were exposed to varying concentrations (10uM, 5uM, and 1uM) of TCS or control. The effects were monitored through five-minute intervals via imaging. As shown in Figure 5, our findings revealed a significant reduction in fluorescent intensity observed in TMRM within thirty minutes post-treatment. Additionally, at higher concentrations - particularly those approaching maximum levels used here - induced mitochondrial fission and swelling primarily occurred at endfeet neural progenitor cell sites where differentiation occurs during neurogenesis.

Discussion

Research has shown that various environmental factors can interfere with neurogenesis resulting in lifelong consequences on brain health. Neurogenesis is a critical process in the development of the central nervous system, and it involves the production, proliferation,

differentiation, migration, and maturation of neural progenitor cells. These processes occur primarily during fetal development when neurons rapidly form synapses and create essential connections. The structural and functional organizations established during this period lay the foundation for future cognitive function. Exposure to TCS is one such factor that affects mitochondrial activity and subsequently causes apoptosis by reducing ATP-dependent respiration. As a result, the unregulated and widespread use of TCS can pose an enormous risk to unsuspecting consumers. This risk is further amplified for infants from exposed populations, potentially leading to cognitive deficits and neural stunting. Elevated concentrations of TCS induced mitochondrial fission and swelling primarily at endfeet neural progenitor cell sites responsible for cell differentiation during neurogenesis within just thirty minutes post-treatment. This is likely an adaptive response to membrane potential loss and the fission pathway's innate protective mechanism in reducing the spread of pathological counterparts [26]. Fission has been shown to directly cause NPCs to no longer proliferate and differentiate into daughter neurons [27]. This reduces the number of potential daughter neurons and overall synaptic density.

Mitochondrial dysfunction disrupts vital physiological processes such as neuronal differentiation and proliferation, which could lead to microcephaly and other developmental disorders. Our findings suggest that even brief exposure to low concentrations may lead to severe toxicity in developing organisms affecting pivotal physiological processes such as neuronal differentiation via modulating specific aspects of energy metabolism linked with mitochondria activity. Specifically, we showed that excessive exposure to TCS causes a significant decrease in TMRM fluorescence intensity due to decreased mitochondrial membrane potentials, as observed through confocal microscopy. Further analysis of mitochondrial activity was assessed using an XFe24 Seahorse Extracellular Flux Analyzer that also showed that overall oxygen consumption

rates, including basal, leak (oligomycin-induced), and maximal oxygen capacity, increased after acute exposure to 30uM of TCS. These increased rates indicate a decrease in ATP-dependent respiration. Where the respiration rates were not directed at the production of ATP but rather a leaky membrane giving way to undirected protons, these results indicate that TCS acts like a mitochondrial uncoupler. As previously discussed, TCS has been found to cause damage to the mitochondrial network and a collapse in membrane potential, as observed through confocal studies. These observations underscore the interconnectedness of mitochondrial function and structure in human pathologies.

Conclusions

The results obtained from these studies highlight the urgent need for a more comprehensive investigation into the long-term effects of TCS exposure on both human development and environmental systems. The available data indicate that such exposure can significantly affect neurogenesis and cognitive performance, thereby underscoring the necessity of further exploration in this field. Subsequent research endeavors should focus on elucidating the underlying mechanisms of TCS toxicity, with particular emphasis placed upon examining its impact on mitochondrial function and structural alterations, to facilitate the identification of potential therapeutic interventions for individuals exposed to this harmful compound. Considering these potential hazards, global regulations must be implemented regarding TCS usage to prevent manufacturers from endangering future generations through their products.

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Figures

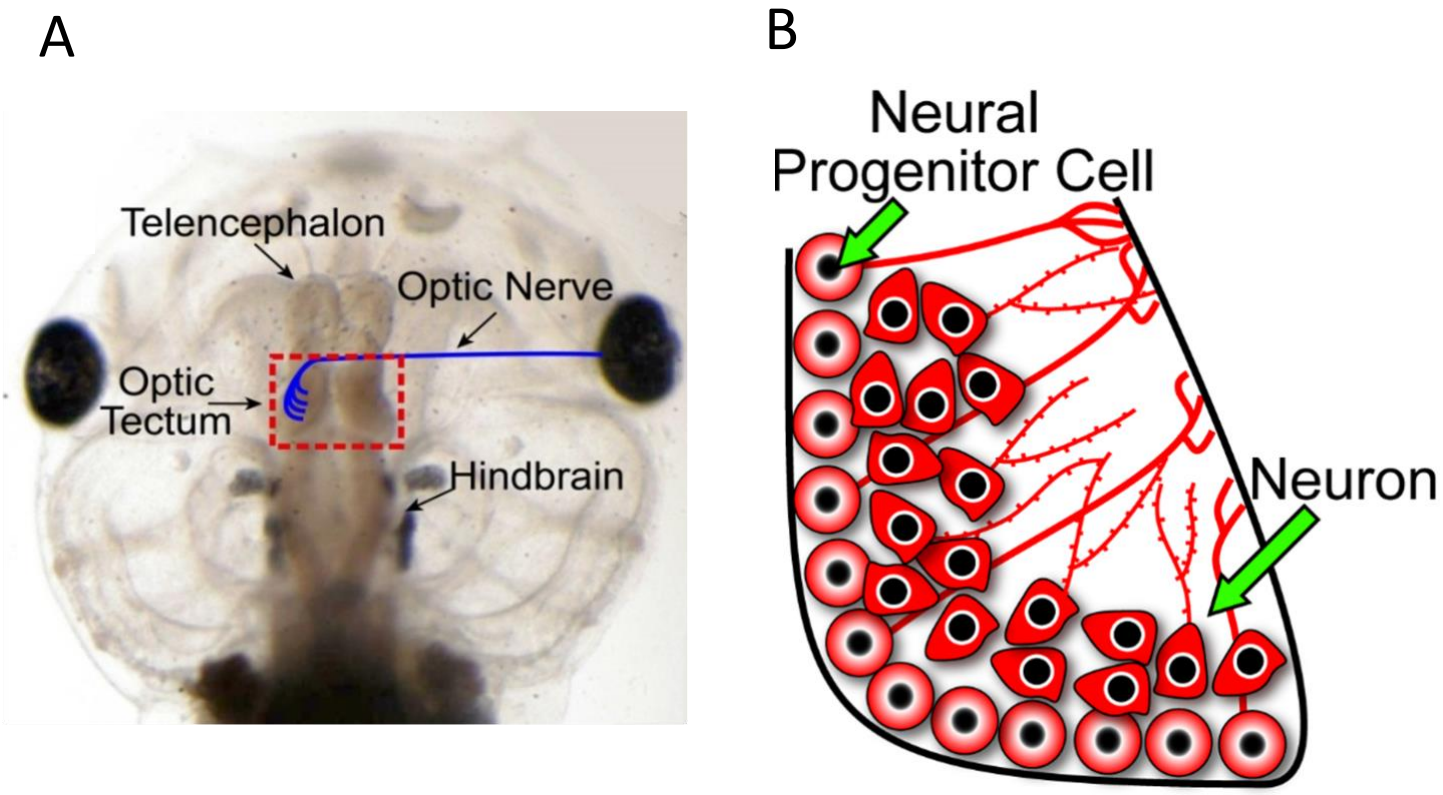


Figure 1: A) Dorsal aspect of the head of a Xenopus laevis tadpole. B) A schematic diagram of the right hemisphere of the midbrain area in the red box in A illustrating the retinotectal circuit. Neural progenitor cells are found in the ventricular proliferative zone. Neurons are generated from NPCs. They extend dendrites into the neuropil, forming synapses with other tectal neurons and/or RGC axons (blue in panel A).

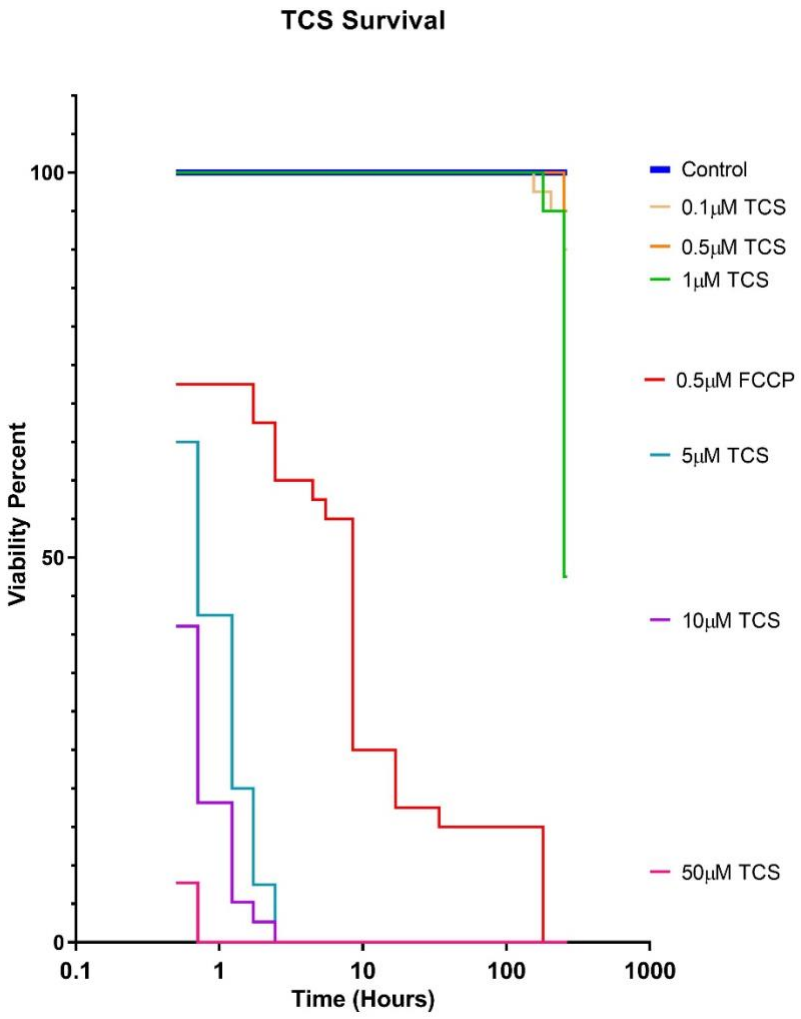


Figure 2: Survival of tadpoles following exposure to TCS and FCCP.

Tadpoles exposed to varying concentrations of TCS and FCCP were monitored for survival rates over 14 days. A prolonged exposure time with 1um TCS resulted in significant mortality on the final day of the experiment; however, negligible effects were observed among tadpoles exposed to Control or lower concentrations (0.5 uM and 0.1 uM) of TCS. Concentrations of TCS at 5um and above proved acutely toxic. While FCCP also exhibited toxicity, tadpoles could tolerate a concentration of 0.5 uM for slightly more extended periods.

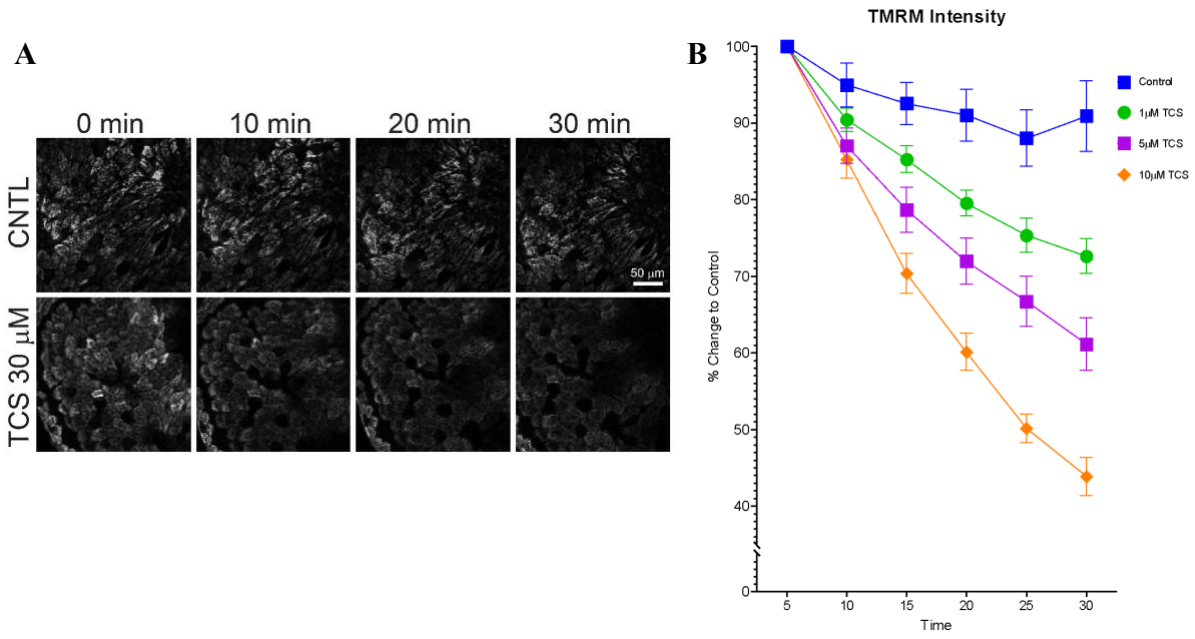


Figure 3: Triclosan (TCS) decreased TMRM intensity in 3-day-old embryos

A) Confocal images of TMRM-labeled skin cells in 3-day-old embryos exposed to either control or TCS 30uM show a dramatic decrease in TMRM intensity upon exposure to TCS, indicating a near complete collapse in mitochondrial membrane potential. **B)** Exposure to lower concentrations of TCS (1 uM, 5 uM, and 10 uM) also reduced mitochondrial function, as evidenced by the decreased intensity of TMRM. 10 uM exhibited roughly a 60% decrease in TMRM intensity within 30 minutes of exposure.

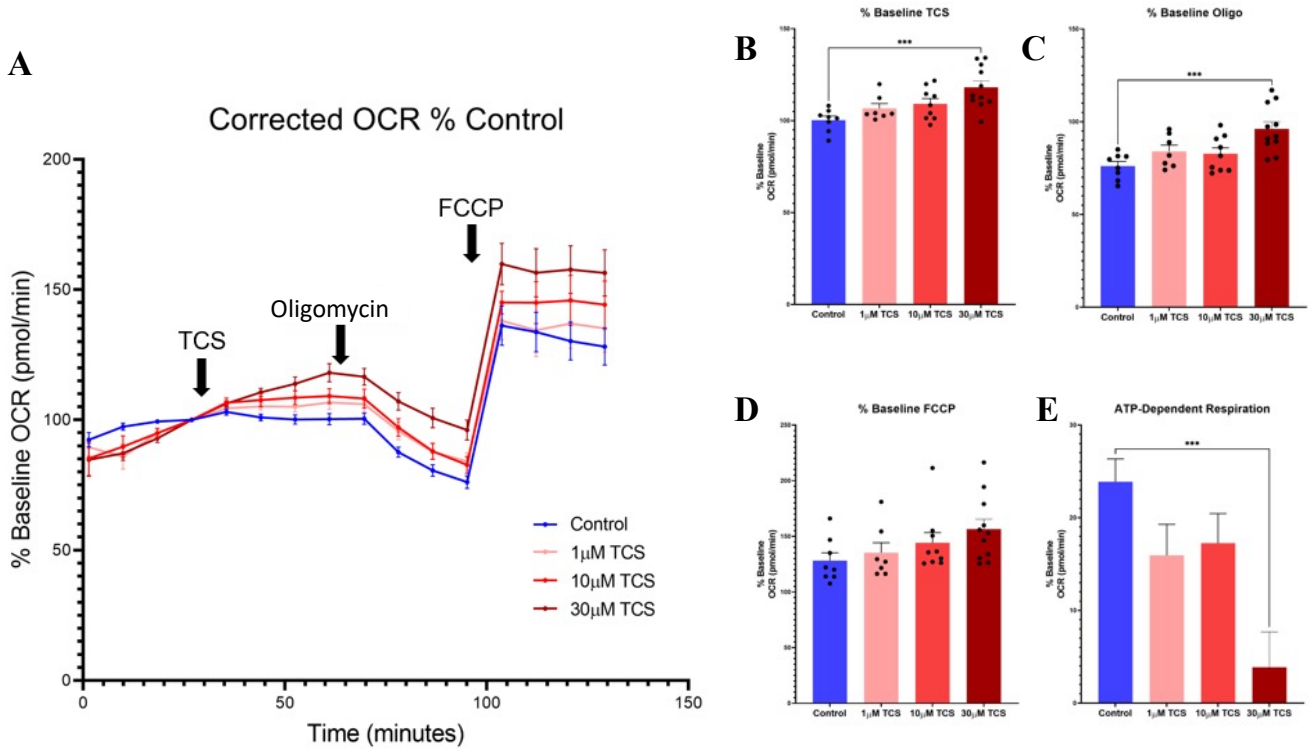
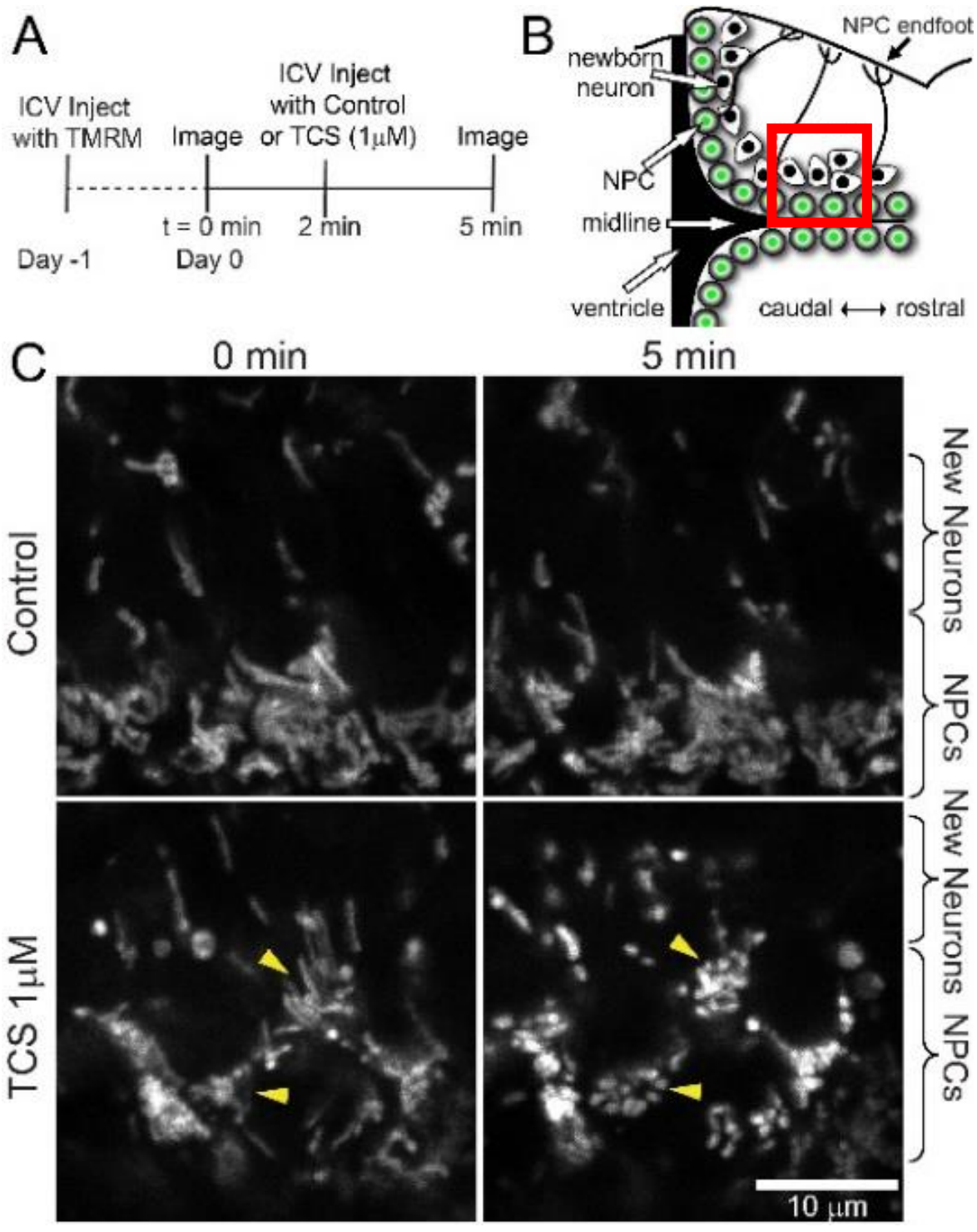


Figure 4: Triclosan increased overall oxygen consumption rate (OCR) but decreased ATP-dependent respiration in whole three-day-old embryos

A) OCR was measured in 3-day-old embryos using a Seahorse Extracellular Flux Analyzer. Tadpoles were exposed to control or different concentrations of TCS, followed by ATPase inhibitor (oligomycin) and inner membrane uncoupler (FCCP). **B)** Tadpoles exposed to 30uM TCS significantly increased (***$p < 0.001$) in OCR following TCS treatment. **C, D)** OCR maintained this significant increase (***$p < 0.001$) in respiration following the ATPase inhibitor, oligomycin (C), and inner membrane uncoupler, FCCP (D). **E)** When ATP-respiration is calculated by subtracting the normalized oligomycin-induced leak respiration from the TCS basal respiration, a significant decrease (***$p < 0.001$) in OCR is depicted. Thus, TCS acts as a mitochondrial uncoupler due to increased basal and leak (oligomycin) respiration, dramatically decreasing ATP-dependent respiration.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TCS 1μM
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Figure 5: Triclosan decreased TMRM intensity in endfeet of neural progenitor cells and induced mitochondrial fission in Stage 47 tadpoles.

We measured TMRM intensity in individual endfeet over time and observed that TCS decreased TMRM intensity. A substantial decrease in TMRM fluorescent intensity is exhibited within 30 min post-treatment. Further, TCS induced mitochondrial fission and swelling at the highest concentrations, particularly in the endfeet of neural progenitor cells. **A)** Tadpoles were injected ICV with TMRM (250 uM). The next day, the ventricular zone was imaged in anesthetized tadpoles. Then the ventricle was injected with either control or 1uM TCS and imaged 3min later. **B)** Illustration showing the imaging location (red box) along the ventricular zone. **C)** Example images showing that TCS induced mitochondrial fission in NPCs (arrowheads) and an apparent increase in TMRM intensity, indicative of an increase in mitochondrial membrane potential.

Chapter V: Conclusions and Future Directions

Mitochondrial dysfunction is a complex and multifaceted phenomenon implicated in the pathophysiology of numerous diseases, including neurodegenerative disorders, metabolic disorders, and cardiovascular diseases. Further research is necessary to fully understand the mechanisms underlying mitochondrial dysfunction and identify effective therapeutic strategies. In chapter two, we highlight the recent research that indicates the interplay between genetic and environmental factors leading to mitochondrial dysfunction, emphasizing the importance of early detection and management of metabolic disorders. We review the studies that indicate that oxidative stress induced by increased reactive oxygen species production significantly promotes mitochondrial dysfunction and exacerbates neurodegenerative disorders like Parkinson's disease and mitochondria myopathies by reducing the capacity of the mitochondria to efficiently produce ATP. Several molecular pathways could be potential targets for developing effective treatment strategies for mitochondrial dysfunction-related diseases. These pathways include enhancing oxidative phosphorylation and ATP production, promoting mitochondrial biogenesis, reducing dysfunctional mitochondria, and regulating the dynamic morphological activity and endogenous antioxidants.

In the studies discussed in chapter three, we showed that the CoQ analog, idebenone, and the potent antioxidant, N-acetylcysteine (NAC), and most notably, the corresponding mitochondrially targeting compounds (MTCs) that are derived from these analogs could help reduce oxidative stress and restore mitochondrial function. These studies utilized cells from a heritable disease, Friedrich ataxia (FRDA), where mitochondrial dysfunction plays a critical role

in the pathogenesis of the disease, providing evidence for the potential of these compounds for therapeutic use.

Comparable mitochondrial dysfunctions may also arise due to inducible illnesses caused by exposure to environmental toxins and lifestyle choices that result in mitochondrial dysfunction. These conditions affect a much larger population and are illustrated in chapter four. We used the in-vivo *Xenopus laevis* model to demonstrate the association between mitochondrial dysfunction and triclosan (TCS), an environmental toxicant in many consumer products. The findings revealed that exposure to TCS resulted in mitochondrial dysfunction, reduced ATP production, and increased mitochondrial fission.

Considering the growing evidence implicating mitochondrial dysfunction in various diseases and conditions, this novel diagnostic approach can be further developed and applied to identify both genetic and environmental sources of mitochondrial dysfunction. Understanding the molecular pathways involved in maintaining function and preventing oxidative damage is crucial to improve treatment strategies for mitochondrial dysfunction. The *Xenopus laevis* model may be used to identify molecules that can mitigate environmental toxin effects, enhance mitochondrial function, and investigate potential therapeutic agents' effectiveness in treating environmental factor-induced mitochondrial dysfunction. The pharmacological enhancement of mitochondrial morphology and function represent novel strategies for treating disorders associated with the environmental and heritable sources of mitochondrial dysfunction. The studies presented in this dissertation highlight the significance of mitochondrial dysfunction in the pathogenesis of various diseases, such as Friedrich ataxia (FRDA) and environmental toxin-induced conditions, and emphasize the importance of developing novel diagnostic methods and treatments targeting both genetic and environmental sources of mitochondrial dysfunction. These studies supported the

hypothesis that various sources of mitochondrial dysfunction result in the same fundamental characteristics: disrupted mitochondrial energy production and formation.

Although our studies offer valuable insights into mitochondrial dysfunction induced by environmental toxins, it is essential to recognize some potential limitations that could impact the generalizability of these findings. Firstly, we used an animal model to simulate human pathogenesis, which may only partially represent some aspects of this complex process, including potential variations in metabolism. Therefore, caution should be exercised when extrapolating these results directly to human populations. Furthermore, different types and levels of toxin exposure may affect mitochondrial function in animals and humans. Hence further investigations are needed to determine whether our findings can be replicated across diverse conditions and species.

Genetic variation may significantly influence individual susceptibility or resilience toward toxicant-induced Mitochondrial Dysfunction. Future studies should also consider how genetic factors interact with environmental exposures leading to mitochondrial dysfunction development and progression. Doing so will help us better understand how genes drive chemical toxicity-related cellular responses and susceptibility while serving as possible predictors for clinical outcomes such as disease risks among exposed individuals.

Understanding the long-term effects of environmental toxicants on mitochondrial health is essential in unraveling the potential epidemiology of mitochondrial diseases. Further research is needed to investigate how molecular pathways control mitochondrial dynamics and their interactions with toxins such as triclosan, which can impact disease pathogenesis. Additionally, more studies are required to enhance diagnostic tools for identifying specific sources that cause mitochondrial dysfunction and evaluating the effectiveness of therapeutic interventions. Overall,

expanding research efforts aimed at elucidating molecular mechanisms underlying mitochondrial dysfunction caused by various agents alongside their interactions with genetics in different population groups would provide much-needed information on tailor-made preventive strategies or treatment options against these ubiquitous yet understudied adverse health outcomes.

In conclusion, mitochondrial dysfunction is a complex and challenging field requiring further investigation to fully understand its health implications. Identifying novel diagnostic approaches and therapeutic strategies highlights the importance of developing tailored treatments for patients suffering from this condition. Our understanding of environmental toxicants' effects on mitochondrial function has significant potential in finding treatment options or preventive measures against these adverse health outcomes. However, there is still a need to expand research efforts focusing on genetic factors contributing to individual susceptibility or resilience toward toxicant-induced mitochondrial dysfunction in development. Furthermore, future studies should explore molecular mechanisms underlying mitochondrial dynamics while assessing their interactions with various toxins and genetics among diverse populations to provide more relevant and personalized therapeutic strategies.