Exogenous Mitochondrial Transfer and IL-6: Modulating Adipocyte Metabolism in Obesity-associated Dysregulation

Soumi Mazumdar

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Exogenous Mitochondrial Transfer and IL-6: Modulating Adipocyte Metabolism in Obesity-associated Dysregulation

Abstract
Obesity, a global health epidemic, poses significant challenges to public well-being due to its complex interplay of metabolic dysregulation, inflammation, and associated comorbidities. Mitochondria, central to ATP production, lipid metabolism, and thermogenesis, play a crucial role in modulating white adipose tissue metabolism. This thesis explores an innovative strategy for addressing mitochondrial dysfunction in obesity which is a critical aspect of this multifactorial problem. The research findings presented herein uncover the impact of introducing exogenous mitochondria into NIH3T3-L1 adipocytes on various aspects of adipocyte function. The key discoveries of this study include a remarkable upregulation of energy expenditure genes, notably Ucp1, Dio2, Ppara, and Ppargc1a, upon exposure to exogenous mitochondria, thereby promoting a favorable shift in metabolic profiles within white adipocytes. Mitochondria therapy also appeared to stimulate mitochondrial biogenesis through an increase in Ppara and Ppargc1a expression. Mitochondria therapy also led to reduced lipid content, indicating a potential means to mitigate adipocyte hypertrophy. Additionally, the release of free fatty acids and glycerol was elevated, reflecting enhanced lipolysis. Thus, this approach showed significant potential for improving energy expenditure and oxidative capacity within adipocytes. Surprisingly, mitochondrial therapy resulted in increased IL-6 levels, contradicting earlier studies showing IL-6 reduction in other injury models. The role of IL-6 in adipose tissue metabolism and obesity has been extensively studied, and this research suggests a possible link between the observed beneficial effects of mitochondria therapy and IL-6 signaling. To test this hypothesis, the study examined the impact of IL-6 neutralization on the metabolic benefits mediated by mitochondrial therapy. Neutralizing IL-6 led to a substantial decrease in the expression of genes associated with energy expenditure, oxidative capacity, and lipid metabolism. This effect highlights the significance of IL-6 in the mechanism of action of mitochondria therapy. In summary, this thesis offers insights into the potential therapeutic implications of mitochondrial transfer in addressing obesity-related metabolic dysregulation. By elucidating specific mechanisms, it contributes to the development of clinically safe treatment options in the ongoing battle against obesity.

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Exogenous Mitochondrial Transfer and IL-6: Modulating Adipocyte Metabolism in Obesity-Associated Dysregulation

Author: Soumi Mazumdar
Advisor: Amandeep Bajwa, PhD

A Thesis Presented for The Graduate Studies Council of The University of Tennessee Health Science Center in Partial Fulfillment of the Requirements for the Master of Science degree from The University of Tennessee in Biomedical Sciences: Molecular and Translational Physiology College of Graduate Health Sciences

December 2023
DEDICATION

In honor of my late father, Sukhendu Bikash Mazumdar, who inspired me to embark on this journey of seeking answers. His enduring influence is woven into every endeavor and all that lies ahead.

To my mother, Ratna Mazumdar, whose unwavering support, and boundless love provide me with the strength to surmount any challenges.

I dedicate this work to both of you as a testament to the promise I have fulfilled.
ACKNOWLEDGEMENTS

I extend my deepest gratitude to my thesis advisor, Dr. Amandeep Bajwa, whose expertise and insight propelled this work forward. Your mentorship has been invaluable, shaping both my scholarly and personal growth. I am indebted to the faculty committee members Dr. Stephen Alway and Dr. Zheng Fan, for their intellectual contributions and encouragement throughout my academic tenure. Their diverse insights have played a vital role in shaping the depth of this work.

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My heartfelt appreciation goes to the members of our lab. Your camaraderie and shared experiences have made this academic journey more meaningful. Each one of you has played a role in shaping the context and environment within which this research was conducted. A special note of appreciation goes to Dr. Maria Namwanje who first conceptualized this project and contributed her dedication and efforts to its early stages. Your vision has laid the foundation for the work presented here. I extend special thanks to Dr. Julia Esswein for her collaborative efforts in co-authoring the literature review, which played a significant role in enhancing the foundation of this research endeavor. I express gratitude to Dr. Laura Sipe for donating the NIH3T3-L1 cells and to Christine Watkins for her invaluable assistance with flow cytometric experiments, instrumental in the experimental aspects of this study.

To my family, friends and peers at UTHSC, your support has been constant amidst the academic ebbs and flows. Your encouragement has been a grounding force.

To those who have shared their knowledge, provided feedback, or offered assistance, I express my gratitude for your contributions.

This thesis serves as a tribute to the combined impact of all who have contributed to my academic journey.
PREFACE

The body of this dissertation is organized in a way that first introduces readers to our rationale for choosing the research topic, objectives, and hypotheses—as well as to present an overview of the literature. A discussion of the materials and methods used then leads to a presentation of the research and final analysis with a discussion of our findings. A concluding chapter relates all research elements back to our final thoughts about the findings and their significance.

For readers to have immediate access to the full presentation of our previously drafted manuscript for publication, the manuscript is presented in the appendix. This mode of presentation allows for Chapter 2, which uses it as its basis, to focus more narrowly on a summary and discussion of this manuscript in the Appendix and to show specifically how it relates to the thesis’ larger goals.

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ABSTRACT

Obesity, a global health epidemic, poses significant challenges to public well-being due to its complex interplay of metabolic dysregulation, inflammation, and associated comorbidities. Mitochondria, central to ATP production, lipid metabolism, and thermogenesis, play a crucial role in modulating white adipose tissue metabolism. This thesis explores an innovative strategy for addressing mitochondrial dysfunction in obesity which is a critical aspect of this multifactorial problem. The research findings presented herein uncover the impact of introducing exogenous mitochondria into NIH3T3-L1 adipocytes on various aspects of adipocyte function.

The key discoveries of this study include a remarkable upregulation of energy expenditure genes, notably Ucp1, Dio2, Ppara, and Ppargc1a, upon exposure to exogenous mitochondria, thereby promoting a favorable shift in metabolic profiles within white adipocytes. Mitochondria therapy also appeared to stimulate mitochondrial biogenesis through an increase in Ppara and Ppargc1a expression. Mitochondrial therapy also led to reduced lipid content, indicating a potential means to mitigate adipocyte hypertrophy. Additionally, the release of free fatty acids and glycerol was elevated, reflecting enhanced lipolysis. Thus, this approach showed significant potential for improving energy expenditure and oxidative capacity within adipocytes.

Surprisingly, mitochondrial therapy resulted in increased IL-6 levels, contradicting earlier studies showing IL-6 reduction in other injury models. The role of IL-6 in adipose tissue metabolism and obesity has been extensively studied, and this research suggests a possible link between the observed beneficial effects of mitochondria therapy and IL-6 signaling.

To test this hypothesis, the study examined the impact of IL-6 neutralization on the metabolic benefits mediated by mitochondrial therapy. Neutralizing IL-6 led to a substantial decrease in the expression of genes associated with energy expenditure, oxidative capacity, and lipid metabolism. This effect highlights the significance of IL-6 in the mechanism of action of mitochondria therapy.

In summary, this thesis offers insights into the potential therapeutic implications of mitochondrial transfer in addressing obesity-related metabolic dysregulation. By elucidating specific mechanisms, it contributes to the development of clinically safe treatment options in the ongoing battle against obesity.
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<th>Description</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>AMP-activated Protein Kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>BAT</td>
<td>Brown Adipose Tissue</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
</tr>
<tr>
<td>CAMK</td>
<td>Calcium/Calmodulin-dependent Protein Kinase</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CL316,243</td>
<td>Synthetic compound, a selective β3-adrenergic receptor agonist</td>
</tr>
<tr>
<td>COX IV</td>
<td>Cytochrome C Oxidase Subunit 4</td>
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<tr>
<td>COX7A1</td>
<td>Cytochrome C Oxidase Subunit 7A1</td>
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<tr>
<td>CYTC</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>Dio2</td>
<td>Deiodinase 2</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>ECMO</td>
<td>Extracorporeal Membrane Oxygenation</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>ESRD</td>
<td>End-Stage Renal Disease</td>
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<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney cells 293T</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutyl Methylxanthine</td>
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<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>NIDDK</td>
<td>National Institute of Diabetes and Digestive and Kidney Diseases</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NIH3T3-L1</td>
<td>Mouse Embryonic Fibroblast Cell Line</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation System</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Solution</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PPARGε1a</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
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<tr>
<td>RAAS</td>
<td>Renin-Angiotensin-Aldosterone System</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription 3</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-alpha</td>
</tr>
<tr>
<td>UCP1</td>
<td>Uncoupling Protein 1</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular Endothelial Growth Factor A</td>
</tr>
<tr>
<td>WAT</td>
<td>White Adipose Tissue</td>
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CHAPTER 1. INTRODUCTION

NOTE: When using Adobe Acrobat, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See Preface for further details.

The Global Obesity Challenge

The twenty-first century has ushered in an era of critical and pressing global health challenges. At the forefront of these concerns is obesity, an epidemic that has reached unprecedented proportions, casting a long shadow over public health. Obesity, characterized by excessive adipose tissue accumulation, has become a major threat to global health, with a considerable effect on morbidity and mortality [1]. World Obesity Atlas 2023 report predicted that half of the global population will develop obesity in the next 12 years unless there are improvements in prevention and treatment strategies (Worldobesity.org). The consequences of this epidemic are far-reaching, affecting not only the physical well-being of individuals but also placing a significant strain on the healthcare system and economic sustainability. Obesity, with myriad health implications, is being recognized as a multifaceted condition that arises from a combination of genetic, environmental, and lifestyle factors. Therefore, addressing the complexities of obesity and developing effective treatment interventions requires a thorough and diverse approach.

The pathophysiology of obesity extends beyond mere fat storage. It involves intricate interactions between adipocytes, metabolic pathways, and inflammatory mediators. Adipocytes, which are the main cellular components of adipose tissue, do not just serve as passive reservoirs for excess energy, but rather play an active role in the maintenance of overall energy homeostasis in the body [2].

The Role of Mitochondria in Obesity

Owing to the multifactorial and complex nature of this condition, conventional therapeutic approaches, such as dietary modification and exercise, while effective to some extent, often fall short. However, in recent years, the role of mitochondria in obesity has gained much attention. Mitochondria, the organelles responsible for cellular energy production, have been identified as important regulators of adipocyte functionality and energy metabolism [3]. These organelles serve as the primary site of ATP generation, chiefly through oxidative phosphorylation, while also participating in the regulation of cellular metabolism, the generation of reactive oxygen species, and the control of apoptosis. Mitochondria are also dynamic in nature and engage in a bidirectional process of fusion and fission, enabling the maintenance of their functional integrity and plasticity.
Mitochondrial dysfunction has been implicated in various metabolic disorders, including obesity, type 2 diabetes, non-alcoholic fatty liver disease and cardiovascular diseases. A strong connection between obesity and mitochondrial dysfunction is becoming increasingly apparent - there is decreased mitochondrial content, impaired oxidative phosphorylation, and changes in mitochondrial structure. Consequently, there has been a growing interest in understanding how manipulating mitochondrial function might offer a promising avenue for mitigating the adverse metabolic effects associated with obesity. To achieve this end, recent research has explored the targeting of mitochondria as a promising approach to alleviate obesity associated metabolic dysfunctions and restoration of energy homeostasis [4-7]. These studies have demonstrated that pharmacological targeting of mitochondria can alleviate pathological oxidative stress, increase mitochondrial biogenesis and result in improvement of overall metabolic profile.

Exogenous Mitochondrial Transfer

Another groundbreaking strategy in addressing metabolic disorders that has garnered attention is exogenous mitochondria transfer, also known as mitotherapy [8]. Exogenous mitochondrial transfer involves the introduction of healthy mitochondria into recipient cells, which have dysfunctional mitochondria. Although the specific mechanisms underlying this phenomenon have yet to be fully elucidated, recent research indicates that exogenous mitochondria can be internalized by target cells, leading to improvements in cellular metabolism and function [9].

Research Questions

It is in this evolving narrative arose some interesting questions - Could the introduction of exogenous mitochondria pave the way to restore metabolic homeostasis in obesity? What effects might this process have on energy expenditure, oxidative phosphorylation, and lipid metabolism within adipocytes? What would be the response of the immune system and inflammatory factors upon introduction of the foreign mitochondria?

Objectives and Aims of the Thesis

In the pages that follow, this thesis investigates the intriguing territory of exogenous mitochondrial transfer as a prospective intervention, elucidating its potential to impact energy expenditure, oxidative capacity, and lipolysis within adipocytes as well as assessed the role of the pleiotropic cytokine IL-6 within this framework.

The in vitro model used in this thesis for studying the effects of exogenous mitochondrial transfer is the NIH3T3-L1 adipocytes, which is a murine origin fibroblast that can be differentiated into mature adipocytes that resemble white adipocytes.
NIH3T3-L1 adipocyte cell line has been widely used as a fundamental model for studying several aspects of adipocyte biology, including adipocyte development and its sensitivity to a wide range of signaling molecules. The differentiation of preadipocytes into mature adipocytes is characterized by significant changes in gene expression, cellular morphology, and functional properties characteristics. As a result, this cell line is an ideal system to investigate the complex metabolic mechanisms that underlie adipocyte function.

A cornerstone of this investigation revolves around the mechanisms of exogenous mitochondrial transfer, delving into the dynamics of this process and assessing the efficacy of mitochondrial internalization. The exploration occurs within the context of comprehensive investigations that center on the fundamental molecular processes and mechanisms involved in mitochondrial transfer.

In addition to examining the mechanics of transfer, this research focuses on the subsequent effects of exogenous mitochondria on the energy expenditure and oxidative metabolism of adipocytes. The focus of this study is in examining the transcriptional response of adipocytes upon the introduction of exogenous mitochondria, and the resulting modifications in the expression of genes that have a direct relationship with energy consumption and oxidative processes. The objective of this study aligns with prior studies that have aimed at explaining the intricate network of mitochondrial regulation of cellular metabolism.

This research aims to expand the scope of investigation in lipid metabolism by examining the impact of exogenous mitochondrial transfer on the adipocyte lipolysis process. The study involves the examination of alterations in lipid accumulation, the release of free fatty acids and glycerol, and changes in the expression of key lipolytic genes. In doing so, it enters a new area of study: the interaction between mitochondrial dynamics and lipolysis, a relationship that underlines the complexities of obesity-related dysregulation.

Furthermore, amid these findings, the role of Interleukin-6 (IL-6), a pleiotropic cytokine, that is dichotomously associated with obesity, takes a prominent position. IL-6, which is produced by several cell types, plays an essential function in the regulation of inflammation and immunological responses. The complex nature of IL-6's involvement in energy metabolism is evident, as it demonstrates both pro-inflammatory and anti-inflammatory actions within the context of obesity. The ambiguous nature of this position brings forward the possibility of being implicated in the regulation of metabolic effects triggered by obesity, particularly in relation to the transfer of mitochondria.

This thesis is structured to address these objectives through a rigorous examination of experimental results, data analysis, and data interpretation. The aim of this study is to provide a scholarly contribution to the expanding field of knowledge in mitochondrial biology, obesity research, and the control of metabolism via cytokines. My specific aims are:
Aim 1  To investigate the effect of exogenous mitochondrial transfer on NIH3T3-L1 adipocytes metabolism, energy expenditure and differentiation.

Aim 2  Assess the role of IL-6 on mitochondrial transfer mediated effects on energy expenditure and oxidative capacity of NIH3T3-L1 adipocytes.

The findings derived from this research have the potential to impact the advancement of novel treatment strategies aimed at addressing mitochondrial function and metabolic dysregulation occurring in obesity and other metabolic disorders.
CHAPTER 2. LITERATURE REVIEW

NOTE: This chapter refers frequently to content in the Appendix. When using Adobe Acrobat, after going there, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See Preface for further details.

Introduction

This chapter summarizes the literature review related to my ETD Aim 1: “To investigate the effect of exogenous mitochondrial transfer on NIH3T3-L1 adipocytes metabolism, energy expenditure and differentiation.” as presented in the review manuscript “Exogenous Mitochondria Transfer: A Promising Therapeutic Approach for Treating Obesity” (Appendix). A summary of the manuscript’s content is as follows:

Summary

This review manuscript discusses an emerging therapeutic approach known as exogenous mitochondrial transfer or “mitotherapy”, which is designed to combat various diseases linked to mitochondrial dysfunction. Mitotherapy involves the transfer or administration of healthy and functional mitochondria to cells or tissues afflicted with mitochondrial impairment. The goal is to restore proper mitochondrial function, thereby improving cellular bioenergetics and overall health.

A central concept in mitotherapy is the idea that healthy mitochondria can replace or supplement dysfunctional ones, offering a promising avenue to tackle a wide range of disorders. This approach leverages the unique properties of mitochondria, such as their ability to undergo fusion and fission, engage in dynamic interactions, and communicate with other cells.

The manuscript delves into the mechanisms underlying the uptake of exogenous mitochondria into recipient cells. Although there isn't a consensus regarding the precise mechanism, proposed theories include endocytosis and macropinocytosis, both of which play a role in engulfing the transplanted mitochondria. Actin dynamics in the cytoskeleton appear to be involved in the uptake process, although more research is needed to clarify the specifics.

A significant portion of the manuscript is dedicated to exploring the rationale for employing exogenous mitochondria transfer in the context of obesity and related metabolic disorders. Research suggests that mitochondrial transfer, particularly from

white adipocytes to white adipose tissue macrophages, plays a vital role in maintaining metabolic homeostasis. However, this transfer can be disrupted by factors like dietary components, such as long-chain fatty acids found in high-fat diets.

The manuscript also highlights various preclinical and clinical models that have explored the potential of mitotherapy. Studies have utilized different sources of exogenous mitochondria, including mesenchymal stem cells, cell lines like HepG2 and HeLa, as well as mitochondria isolated from tissues like liver and skeletal muscles. The source of mitochondria has implications for safety and effectiveness in clinical applications, and various delivery methods, such as direct injection and intravenous administration, have been assessed.

Furthermore, the manuscript underlines the importance of safety and efficacy in clinical settings. Ethical considerations, genetic compatibility, and screening for contaminants are crucial factors to be addressed when considering mitotherapy as a treatment option. While some clinical success has been achieved, the search for less invasive delivery methods continues.

In conclusion, the manuscript provides a comprehensive overview of mitotherapy and its potential applications in addressing obesity and associated metabolic disorders. The central theme revolves around the transfer of healthy mitochondria to restore proper mitochondrial function, enhance cellular energy metabolism, and mitigate the effects of disorders like non-alcoholic fatty liver disease and insulin resistance. However, challenges related to safety, efficacy, and delivery methods underscore the need for further research, including clinical trials and studies in large animal models, to establish Mitotherapy as a viable therapeutic approach in the realm of metabolic health.

**Role of IL-6 in Adipose Tissue Metabolism**

The observed effects of mitochondrial therapy on adipose tissue metabolism introduce a novel approach to tackling obesity. Exogenous mitochondria have the potential to induce metabolic changes that mitigate lipid accumulation, enhance lipolysis, and stimulate mitochondrial biogenesis. But this promising strategy comes with a caveat: the role of IL-6.

During this study, a possible role of IL-6 in this process became evident. However, further investigation is required to fully comprehend the extent of its influence and the precise mechanisms through which it either supports or hinders exogenous mitochondria therapy.

From a historical perspective, Interleukin 6 (IL-6) has predominantly been regarded as a cytokine with pro-inflammatory properties [10]. It has been implicated in the pathogenesis of obesity-associated inflammation as a contributor to insulin resistance and metabolic dysfunction, a perception that has significantly influenced the scientific community's understanding of the topic [11-13]. IL-6 secreted by white adipose
tissue has been shown to be responsible for recruitment and activation of macrophages within the adipose depots, leading to a cascade of inflammatory events [14]. Macrophages infiltrating the expanding adipose tissue release pro-inflammatory such as cytokines, including IL-6 and TNF-α, thereby continuing this insidious cycle [15]. This chronic low-grade inflammation contributes to insulin resistance, a key feature of obesity [16].

However, IL-6's role in obesity is far from unidimensional. One of the fascinating aspects of the role of IL-6 in adipose tissue metabolism, is its ability to promote the phenotypical transformation of white adipose tissue (WAT) into brown adipose tissue (BAT)-like characteristics, a process referred to as “browning”. This transformation holds the promise of increased energy expenditure and thermogenesis [17]. A study by Buzelle et al has demonstrated that IL-6 plays a role in mediating the effects of CL316,243, a beta-adrenergic agonist in vivo [18]. The CL316,243 mediated increase in energy expenditure observed in wild type mice was found to be attenuated in the IL-6 knockout mice.

Another facet of the paradoxical role of IL-6 is its influence on lipid and glucose metabolism within adipocytes. It has been demonstrated that IL-6 aids in insulin stimulated glucose homeostasis and lipid metabolism both in vitro and in vivo [19]. Furthermore, IL-6 can induce lipolysis and increase fat oxidation [20]. This is further corroborated by a recent study that has shown that blocking IL-6 prevents the mobilization of free fatty acids in both lean and obese individuals [21]. Prolonged IL-6 administration has also been found to have enhanced fatty acid oxidation while decreasing triglyceride accumulation in liver and skeletal muscle [22]. This ability of IL-6 is vital in maintaining lipid metabolism and preventing excessive fat accumulation, which is often associated with metabolic disorders like obesity.

Another significant role played by IL-6 is its impact on mitochondrial biogenesis and function. Mitochondrial biogenesis is the process through which new mitochondria are generated within cells, increasing their overall mitochondrial content. Recent studies have revealed that IL-6 can influence mitochondrial biogenesis via AMPK signaling pathways [23, 24]. One of the pathways through which IL-6 impacts mitochondrial biogenesis is the signal transducer and activator of transcription 3 (STAT3). IL-6 activates STAT3, which translocates to the nucleus and interacts with Ppargc1a, ultimately leading to the upregulation of mitochondrial genes [25, 26]. This activation of mitochondrial biogenesis supports an increased capacity for nutrient utilization and energy production, making IL-6 a significant player in metabolic regulation [27].

One crucial aspect of mitochondrial function is the regulation of oxidative phosphorylation, a process that occurs in the mitochondrial electron transport chain (ETC) to produce ATP. Dysregulation in this process can result in oxidative stress and decreased energy production [28]. IL-6 has been found to enhance ETC activity and increase ATP production as well as protect against oxidative stress [29, 30]. This boost in ATP generation contributes to enhanced cellular energy, which is particularly useful in muscle cells during exercise and energy-demanding situations [31].
The actions of IL-6 in adipose tissue have also been shown to be dependent on source and the signaling pathways involved [32-34]. This multifaceted signaling molecule engages in a dual mechanism, which includes both classic and trans-signaling pathways to manifest its effects. In the classical signaling pathway, IL-6 binds to the transmembrane IL-6 receptor (IL-6R) located on selective target cells such as neutrophils, naive T cells, and hepatocytes [35]. The IL-6/IL-6R complex then binds with the transmembrane signal-transducing glycoprotein 130 (gp130), initiating a cascade of downstream signaling events [36, 37]. Beyond the conventional pathway, it also operates through a mechanism known as "trans-signaling." In this alternative route, IL-6 binds to its soluble receptor, resulting in the formation of a soluble complex. This complex can then interact with the widespread gp130, which is expressed in almost all cells of the body [38]. This distinctive feature allows the IL-6/sIL-6 complex to exert its signaling influence across various cell types and tissues.

This intricate network of IL-6 signaling pathways underscores the crucial role of IL-6 in diverse physiological processes. The ultimate impact of IL-6 signaling depends on a nuanced interplay of factors: the specific target cell type, its distinct intracellular environment, and the concurrent external stimuli surrounding the cell [32]. The broad-ranging effects of IL-6 are a testament to its pleiotropic nature. Consequently, IL-6 finds itself entwined in critical physiological systems, including immune regulation, where it can exhibit both pro- and anti-inflammatory effects. Furthermore, it plays important roles in energy homeostasis, lipid and glucose metabolism as well contribute to bone metabolism, the central nervous system, as well as act as an endocrine regulator, reflecting its indispensable role in maintaining overall bodily homeostasis [39].

One of the most pressing issues in obesity research is whether IL-6 may be used as a therapeutic target. Early evidence suggested that IL-6 suppression could be beneficial due to its role in inflammation and insulin resistance. However, the emergence of its browning and metabolic effects adds complexity to this therapeutic landscape. Various studies have explored IL-6 inhibitors, such as monoclonal antibodies, for the treatment of inflammatory conditions, including rheumatoid arthritis [40, 41].

While the central focus of this study remains the evaluation of therapeutic potential of mitotherapy in obesity, an investigation into the role that IL-6 might be playing in it becomes essential to fully elucidate the underlying mechanisms and explore potential clinical applications. As our understanding of this complex relationship continues to evolve, it will open new avenues for investigating novel treatments and preventive strategies for metabolic disorders.
CHAPTER 3. METHODS AND MATERIALS

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Study Design

NIH3T3-L1 cells were cultured in 12-well plates, allowed to reach confluence, and subsequently differentiated into mature adipocytes. The cells were treated on day 8 of differentiation. For the mitotherapy experiments, the cells were treated in four different groups- (1) isolated mitochondria (10 µg/mL), (2) CL316,243 (10 µM), (3) a combination of both mitochondria and CL316,243, and (4) a control group treated with PBS as a vehicle. CL316,243 is a synthetic compound known for its role as a selective β3-adrenergic receptor agonist. By activating these receptors, CL316,243 promotes thermogenesis and increases energy expenditure in adipose tissue, due to which this compound has been widely employed in scientific studies investigating the regulation of energy balance and potential applications in the treatment of obesity and related metabolic disorders. CL316,243 is added at the same time as exogenous mitochondria, both when added as separate treatment groups as well as when combined. For the experiments with both mitochondria and IL-6, cells were treated with either control IgG antibody or anti-IL-6 IgG antibody (5 µg/mL) one hour prior to exposure to either PBS or exogenous mitochondria (10 µg/mL). Cells were maintained at 37°C with 5% CO2 and harvested after 72 hrs of treatment, unless mentioned otherwise. A schematic demonstrating the method is presented in Figure 3-1.

Cell Culture and Differentiation

Mouse embryonic fibroblasts (NIH3T3-L1) cells were passaged in DMEM containing 10% Bovine Calf Serum, seeded in 6 well or 12 well plates and grown to confluence. The cells were cultured for two additional days post-confluence in the DMEM containing bovine calf before induction of differentiation. On day 0 of differentiation, cells were switched to a DMEM media containing 10% Fetal Bovine Serum (FBS), 0.05 mM Isobutyl methylxanthine (IBMX) (Sigma-Aldrich I7018), 1 µM Dexamethasone (Sigma-Aldrich, D4902), and 10 µg/ml bovine insulin (Sigma-Aldrich, I6634). On day 2 after differentiation initiation, the induction media was replaced with fresh DMEM containing 10 µg/mL insulin. Media was changed every other day with DMEM containing 2.5 µg/mL insulin until cells displayed characteristics of fully differentiated mature adipocytes on Day 8 (characterized by round cells with visible lipid droplets under a light microscope). Only cells with at least 90% differentiation were used in the experiments.
Figure 3-1. Study design and methodology.
Mitochondrial Isolation and Assessment of Purity

For isolation of mitochondria, healthy HEK293T cells with GFP labelled mitochondria were used. For the labelling, a GFP tag was fused to cytochrome oxidase C following a previously described protocol [42]. Cells were grown in 15 cm plates until they were 90% confluent. 5 ml of homogenizing buffer containing 300 mM sucrose, 1mM EGTA, 10mM HEPES and 40 mg/ml subtilisin is added to each plate and incubated at room temperature for 5 mins. After that, all the buffer containing cell suspensions are transferred to a pre-chilled 50 ml conical tube and incubated on ice for 15 mins. Subsequent steps were carried out at 4°C, with all tubes maintained on ice. The cell suspension was first centrifuged at 500 x g for 5 mins. The supernatant was then sequentially filtered through two 40 µm filters and one 10 µm filter, with the pellet discarded. The suspension was subsequently centrifuged at 800 x g for 5 mins, and the supernatant was collected in a fresh conical tube. Finally, after centrifugation at 3500 x g for 10 mins, a pellet containing isolated mitochondria was obtained, which was resuspended in sterile 1X Phosphate Buffered Solution (PBS). The protein concentration of the mitochondrial fraction was quantified using the Bradford Assay, and the functional mitochondria estimation was performed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7571). Image of freshly isolated GFP tagged mitochondria is depicted in (Figure 3-2).

The purity of isolated mitochondria was further assessed by analyzing protein expression of β-tubulin and Cox IV in the whole cell extract and mitochondrial fraction (Figure 3-3). β-tubulin was expressed solely in the whole cell extract and absent in the mitochondrial fraction. Additionally, Cox-IV which is present in the inner mitochondrial membrane was expressed in both, however more intense band was observed in the mitochondrial fraction.

Mitochondria Treatment

The pelleted isolated mitochondria were resuspended in 200-250 µl of sterile 1X Phosphate Buffered Solution (PBS). The protein concentration of the mitochondrial fraction was quantified using the Bradford Assay, and the functional mitochondria estimation was performed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7571).

The isolated mitochondria were kept on ice until treatment. For most of the experiments, unless mentioned otherwise, a 10 µg/ml dosage of mitochondria was used which was added to the cell culture media and mixed gently before adding on to the plates. An equivalent volume of sterile 1X PBS was used as vehicle.
Figure 3-2  Isolated GFP tagged mitochondria visualized by EVOS FL auto imaging system (scale bar= 400 µm).

Figure 3-3  Western blot analysis confirms purity of isolated mitochondrial fraction.
WC= whole cell, Mito=mitochondria
Staining and Imaging

NIH3T3-L1 cells were seeded in 6-well plates and treated for 72 hrs in one of four treatment groups: vehicle, CL316,243, exogenous mitochondria, or a combination of both. Subsequently, cells were washed with 1X PBS and fixed with 4% paraformaldehyde for 15 mins. After fixation, cells were washed with deionized water, stained with Oil Red O for 30 mins, and washed thrice with deionized water. Hematoxylin eosin stain was applied for 1 min, followed by 3-5 additional washes with deionized water. The cells were imaged using the EVOS FL Auto imaging system at 10X and 20X magnifications. Oil Red O stain was extracted by washing with 100% isopropanol for 10 mins, and colorimetric optical density (OD) was measured at 492 nm using Agilent BioTek Synergy HTX Multi-Mode Microplate Reader.

Gene Expression Analysis

RNA was extracted using the Direct-Zol Miniprep Plus kit (Zymo Research, R2072) and its concentration was quantified. Then, cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad, 1708841). After cDNA synthesis, the samples along with forward and reverse primers of relevant genes, iTaq Universal SYBR Green Supermix (Bio-Rad, 1725124) and nuclease free water were added to a 96-well qPCR plate. Along with 1 μl of forward and reverse primer each, 10 μl of SYBRgreen mix and 3 μl of nuclease free water such that the final volume in each well becomes 20 μl. qPCR was performed using the QuantStudio 3 Real-time PCR system (Applied Biosystems). Gene expression levels were calculated using the ΔΔCt method with cyclophilin (CypA) as an internal reference control.

Protein isolation and Western Blot: After 72 hrs of treatment, media was removed, and RIPA buffer and protease/phosphatase inhibitor were added to each well. Cells were sonicated, and the resulting supernatant containing cellular proteins was transferred to a sterile tube. Protein concentration was quantified using the Bradford assay. 30 μg of protein of each sample was loaded on to a NuPage 4-12% Bis-Tris gel and, the proteins were transferred to a PVDF membrane. The membranes were blocked with 5% BSA in 1X TBST and incubated in primary antibody (1:1000) overnight at 4 C. Next day, the membranes were washed and then incubated in secondary antibodies (1:10000) for an hour. Protein signals were visualized by chemiluminescence with SuperSignal West Pico PLUS chemiluminescent substrate (Invitrogen, 34578) and quantified using ImageJ Software. Antibodies used are as follows: rabbit anti-UCP1 (Abcam, ab10983), mouse anti β-actin (AC-15) (Invitrogen, MA1-91399), rodent OXPHOS cocktail (Abcam, ab110413), rabbit anti-COX IV (Abcam, 3E11), mouse anti β-tubulin (Abcam, ab6046) and secondary anti-mouse (Santa Cruz, 516102) and anti-rabbit (Cell Signaling, 7074) antibodies.
Lipolysis Assay

Lipolysis was assessed by quantifying the amount of free fatty acids and glycerol in the cell culture media induced by different treatments. For this the mature adipocytes were treated with exogenous mitochondria, CL316,243, both mitochondria and CL316,243 or vehicle in a DMEM media containing 2% fatty acid-free BSA. Media was collected at timepoints of 6, 24, 48, and 72 hrs for measurement of free fatty acids (Abcam, ab65341) and free glycerol (Abcam, ab65337).

ELISA

Cell culture media collected at timepoints of 6, 24, 48, and 72 hrs after experimental treatments were subjected to ELISA assays to measure the levels of IL-6, TNF-α, and IL-10 using mouse ELISA kits (Invitrogen, 88-706, 88-7324-88, 88-7105-88) following the manufacturer’s protocol.

Flow Cytometry

To confirm successful differentiation of NIH3T3-L1 preadipocytes into mature adipocytes, cells were stained with Bodipy 493/503 to visualize lipid droplets and incubated for 20 mins at 37°C. Unstained negative controls were prepared for both preadipocytes and adipocytes. Cells were washed with PBS, trypsinized, and resuspended in 1X PBS for flow cytometric analysis. To assess mitochondrial uptake, cells were treated with 0 μg, 5 μg, 10 μg, 20 μg and 50 μg of exogenous GFP tagged mitochondria (isolated from HEK cells) per ml of media.

Prior to starting all cells were stained with Propidium iodide for 5 mins to distinguish live cells from dead ones. The stained cells were analyzed by using a MacsQuant Analyzer 16 (Miltenyi). FlowJo software was used to analyze lipid content and mitochondrial uptake.

Statistical Analysis

All experiments were independently repeated 3 times and all conditions done in triplicate. Data were analyzed using GraphPad Prism 9.3.1 (GraphPad Software, La Jolla California USA) statistical significance was determined at p ≤ 0.05. Data points are presented as mean ± standard error of the means (SEM). Statistical tests included unpaired 2-tailed Student’s t-tests, one-way analysis of variance (ANOVA), and two-way ANOVA, as well as multiple comparison tests as appropriate.
CHAPTER 4. RESULTS

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Exogenous Mitochondria are Successfully Taken up by Mature Adipocytes

Prior to commencing the mitotherapy experiments, the validation of successful differentiation from NIH3T3-L1 preadipocytes to mature adipocytes was rigorously assessed through light microscopy, maturation gene expression analysis, and flow cytometric quantification of cellular lipid content (Figure 4-1). For experimental utilization, it was ensured that at least 90% of the cells were successfully differentiated (Figure 4-1A). Eight days after the initiation of the differentiation protocol, the significant upregulation of adipocyte maturation-related genes, including Adipoq, Adipisin, Pparg, and Lpl, served as evidence of the full transition of the preadipocytes into mature adipocytes (Figure 4-1B). This was further substantiated by flow cytometric analysis, which involved intracellular neutral lipid staining with Bodipy, revealing a remarkable fifteenfold increase in lipid content within the differentiated cells compared to their preadipocyte precursors (Figure 4-1C).

Twenty-four hours following treatment, the successful internalization of exogenous mitochondria by mature adipocytes was confirmed through microscopic examination (Figure 4-1D). As illustrated in, the presence of GFP-tagged mitochondria was distinctly observed within the cell, as evidenced by the vibrant green fluorescence closely localized to the Hoechst-stained nuclei of the cells, which appeared blue.

Furthermore, it was found that there was a dose-dependent relationship with the amount of mitochondrial uptake. The flow cytometric study of live, mature adipocytes treated with GFP-tagged exogenous mitochondria indicated that uptake grew incrementally corresponding to the escalating dose of administered mitochondria. Specifically, 2-fold, 3-fold, 8-fold and 13-fold elevation in FITC A fluorescent intensity was observed in cells treated with 5 µg, 10 µg, 20 µg, and 50 µg of mitochondria, respectively, when compared to the vehicle-treated controls (Figure 4-1E).

Treatment with Healthy Mitochondria Increases Expression of Energy Expenditure, Mitochondrial Biogenesis and Oxidative Genes in Mature Adipocytes

Upon exposure to exogenous mitochondria for a duration of 72 hrs, a significant upregulation in the expression of key genes associated with energy expenditure became evident. Notably, the expression of Ucp1 within the mitochondria-treated group exhibited a 10-fold augmentation compared to the vehicle-treated control. This marked
Figure 4-1  Exogenous mitochondria were successfully taken up by mature adipocytes in a dose dependent manner. 
(A) EVOS images showing NIH3T3-L1 cells during differentiation into adipocytes (scale bar = 400 µm). (B) Increased expression of adipocyte maturation genes (relative to cyclophilin A), indicating successful differentiation. (C) Histogram of flow cytometric analysis showing increased FITC-A signal in the differentiated adipocytes indicating higher lipid content compared to preadipocytes and quantification of FITC-A signal expressed as mean fluorescence intensity (MFI). (D) Brightfield image of the NIH3T3-L1 adipocytes, GFP image showing GFP tagged mitochondria and merged image showing exogenous mitochondria in the cells (scale bar=200 µm). (E) Histogram of flow cytometric analysis showing FITC-A signal from the varying doses of GFP tagged exogenous mitochondria in the treated adipocytes and quantification of the FITC-A signal as observed in different doses. Data are presented as mean±SEM in each experimental group (n=6) and *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
elevation was followed by respective increases of 5-fold, 3-fold, 1.5-fold, and 2-fold in the expression levels of Dio2, Elovl3, Ppara, Ppargc1a and Prdm16, respectively. When compared to the vehicle, these transcriptional improvements were reflected in the protein expression of UCP1 in the mitochondria-treated group which showed a 1.5-fold increase (Figure 4-2A).

Concurrently, the expression of genes associated with oxidative metabolism, such as Cox7a1, CytC, and Cox8b, was found to be elevated in the mitochondria-treated group as compared to the control group, with fold-increases of 4, 2.5, and 2.5, respectively. Furthermore, on comparing OXPHOS complex protein expression levels between the vehicle and mitochondria treated groups, a significant elevation was observed in Complex I expression with almost 15-fold increase. A similar trend was observed in the protein expression of the remaining complexes where the mitochondria treated groups showed higher expression compared to the vehicle group (Figure 4-2B).

Mitochondrial Transfer Augments Lipolytic Activity in Adipocytes

The treatment of adipocytes with exogenous mitochondria resulted in a notable reduction in intracellular lipid content, as assessed by Oil Red O staining (Figure 4-3A). This decrease in lipid accumulation correlated with an augmented lipolytic response induced by the transfer of mitochondria to adipocytes. Following mitochondrial transfer there was a marked increase in the release of free fatty acids at both the 6-hr and 72-hr timepoints, as well as an increase in the release of glycerol at the 6-hrs and 24-hrs timepoints (Figure 4-3B and C).

Moreover, gene expression analysis of key lipolytic genes, notably Atgl, Lipe, and CD36 revealed a robust upregulation. Specifically, Atgl, Lipe and CD36 expressions exhibited a 2.5-fold, 3-fold and 7-fold increases respectively (Figure 4-3D). This underlines the enhancement of lipolytic activity because of mitochondrial transfer, with a concomitant alteration in the expression of genes implicated in lipid metabolism.

Mitochondrial Transfer Diminished Expression of Adipogenic Genes in Adipocytes

The transfer of exogenous mitochondria elicited a downregulation of adipogenic genes when compared to the vehicle-treated group. A significant reduction was observed in the gene expression levels of key adipocyte maturation regulators, including Adipoq, Adipsin, and Pparγ, all of which play critical roles in the adipocyte differentiation process (Figure 4-4).

When considered in the context of the observed morphological changes in the adipocytes after exogenous mitochondria treatment, this novel and intriguing finding prompts the consideration that mitochondria therapy may cause de-differentiation like phenomenon in the adipocyte population.
Figure 4-2  Treatment with healthy mitochondria increases expression of energy expenditure, mitochondrial biogenesis, and oxidative genes in mature adipocytes. (A) Real-time qPCR analysis of gene involved in energy expenditure & mitochondrial biogenesis (relative to Cyclophillin A) (n=10) and western blotting analysis of UCP1 (relative to β-actin) and quantification of protein expression (n=6). (B) Real-time qPCR analysis of oxidative genes (relative to cyclophilin A) (n=6), western blot analysis of OXPHOS complex relative to VDAC and quantification of protein expression(n=3), data are presented as mean±SEM and *p ≤ 0.05, **p ≤ 0.01
Figure 4-3 Mitochondrial transfer augments lipolytic activity in adipocytes.

(A): Changes in 3T3-L1 cell morphology – top panel-brightfield, middle panel- Bodipy 493/503 and bottom panel- Oil Red O + H&E (scale bar=200 um) and quantification of Oil Red O absorbance after 72 hrs of treatment, (B) Free fatty acid, (C) Free glycerol content present in the culture media measured by colorimetric assay, (D) qPCR analysis of genes involved in lipid metabolism in cells after 72 hrs of treatment (relative to cyclophilin A), data are presented as mean±SEM in each experimental group (n=6) and *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Figure 4-4  Mitochondrial transfer diminished expression of adipogenic genes in adipocytes.
qPCR analysis of genes involved in adipocyte maturation in cells after 72 hrs of treatment (relative to cyclophilin A). Data are presented as mean±SEM in each experimental group (n=6) and *p ≤ 0.05, **p ≤ 0.01.
Mitochondrial Transfer Induced IL-6 Levels

Given the link between obesity, macrophage recruitment, and the release of inflammatory cytokines that contribute to the exacerbation of insulin resistance and metabolic dysfunction, it becomes imperative to evaluate the impact of exogenous mitochondria transfer on cytokine levels in an in vitro setting.

Interestingly, among the cytokine genes including Il-6, Tnf-α, and Il-10, only Il-6 expression within the mitochondria-treated group was significantly higher than that in the control group. (Figure 4-5A).

Furthermore, cytokine assays conducted after 72 hrs of treatment corroborated these observations, since only IL-6 protein levels in the cell culture media of the mitochondria-treated group was significantly higher than that in the control group. The peak of IL-6 protein levels was observed at the 6-hrs timepoint following the mitochondria treatment, with a gradual reduction noted at the 24-hrs and 72-hrs timepoints. (Figure 4-5B).

IL-6 Neutralization Significantly Reduced Energy Expenditure and Oxidative Genes Following Mitochondrial Transfer

To elucidate the role of IL-6 in the mitochondria-induced enhancement of energy expenditure and oxidative capacity in adipocytes, exogenous mitochondria were introduced in the presence of either a control IgG antibody or a neutralizing IL-6 monoclonal antibody. The efficacy of the neutralizing antibody was established through ELISA, which confirmed a substantial decrease in IL-6 protein levels across all timepoints in both the vehicle-treated and mitochondria-treated groups (Figure 4-6A).

The neutralization of IL-6 combined with mitochondria treatment resulted in a drastic reduction in the expression of energy expenditure genes and oxidative genes in comparison to cells treated with the IgG control antibody. This reduction was particularly pronounced in the case of Ucp1, Dio2, Cox7a1, CytC, Ppara, and Ppargc1a gene expressions (Figure 4-6B).

The expression of Ucp1, a critical gene associated with energy expenditure, exhibited a highly significant increase upon mitochondrial treatment compared to the vehicle-treated group. However, when IL-6 was neutralized, the considerable rise in Ucp1 expression was significantly decreased. Furthermore, in the presence of the IL-6 antibody, UCP1 protein expression was shown to be partially diminished in the mitochondria-treated group. Collectively, these findings are strongly suggestive of a model in which the production of IL-6 following mitochondrial treatment plays a crucial role in augmenting energy expenditure and oxidative capacity of the NIH3T3-L1 adipocytes (Figure 4-6C).
Figure 4-5  Mitochondrial transfer induced IL-6 levels.

(A) qPCR analysis of cytokine gene expression in treated adipocytes after 72 hrs (relative to cyclophilin A) (n=10) (B) Cytokine levels in the culture media measured by ELISA at 6, 24 and 72 hrs after treatment (n=6), data are presented as mean±SEM and *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤0.0001.
Figure 4-6   IL-6 neutralization significantly reduced energy expenditure and oxidative genes following mitochondrial transfer

A) Neutralizing effect of IL-6 monoclonal antibody was confirmed by measuring IL-6 levels in cell culture media by ELISA at 24, 48 and 72 hrs after treatment (n=3), (B) Relative expression of energy expenditure and oxidative genes (relative to cyclophilin A) (n=6), and (C) Western blot analysis of UCP1 expression in adipocytes treated with IgG or IL-6 Ab 1 hour prior to mitochondria treatment for 72 hrs (n=2), data are presented as mean±SEM and *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤0.0001
CHAPTER 5. DISCUSSION AND CONCLUSIONS

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The persistent and widespread occurrence of obesity on a global scale remains a significant and formidable obstacle to the promotion of public health and overall well-being. Obesity is a condition characterized by an imbalance in energy metabolism, excess lipid accumulation, insulin resistance and systemic inflammation \[43\]. These physiological changes are interconnected and give rise to a cascade of associated comorbidities, including type 2 diabetes, non-alcoholic fatty liver disease and cardiovascular diseases \[44\]. Given the current circumstances, it is evident that there exists a pressing demand for novel therapeutic interventions that effectively tackle the intricate dynamics at play within these processes. Furthermore, mitochondrial dysfunction in obesity gives rise to excessive reactive oxygen species generation which contributes to the pathological process. Therefore, lowering the oxidative stress levels induced by mitochondrial dysfunction in obesity has also been found to be a key component of therapeutic strategy against obesity \[45\].

Mitochondria play central roles in important metabolic processes including ATP production, lipid metabolism, fatty acid oxidation and thermogenesis. The promotion of WAT “browning”, a transformation of WAT that confers its BAT like characteristics, has been shown as one of the key strategies targeting mitochondria for metabolic disease management \[46\]. By increasing the thermogenic capacity of adipose tissue, browning contributes to the dissipation of excess energy as heat and the mobilization of stored lipids.

One of the major findings of this study is that the introduction of exogenous mitochondria into NIH3T3-L1 adipocytes results in a significantly increased expression of energy expenditure genes, including Ucp1, Dio2, Prdm16, Ppara and Ppargc1a. Ucp1, an important mediator of thermogenesis and energy expenditure in brown adipocytes, is known to play a key role in combating obesity by promoting the dissipation of energy as heat \[47\]. The significant increase in Ucp1 expression in response to mitochondrial therapy points towards the potential of this approach to increase energy expenditure, thereby leading to a favorable alteration in the metabolic profile of the white adipocytes.

Mitochondrial biogenesis is the process by which new mitochondria are generated within cells, enhancing their overall mitochondrial content. This process is modulated by Ppara and Ppargc1a along with other transcriptional coactivators, representing a potential therapeutic target. Studies have demonstrated that the upregulation of Ppargc1a and related factors through pharmacological interventions or lifestyle modifications can increase mitochondrial biogenesis. This is particularly beneficial for adipocyte
metabolism as a higher number of mitochondria translates to an enhanced capacity for nutrient utilization and thermogenesis. The increase in Ppara and Ppargc1a induced by mitotherapy suggests that exogenous mitochondria may be exerting stimulatory effects on the mitochondrial biogenesis pathway at some capacity. However, further exploration is required to understand this mechanism better.

Mitochondria are essential for lipid metabolism and fatty acid oxidation. They play a critical role in breaking down fatty acids into acetyl-CoA, a key component of the citric acid cycle, ultimately leading to ATP production. Dysfunction in this process can lead to lipid accumulation and metabolic perturbations. Thus, targeting mitochondria to enhance lipid metabolism and fatty acid oxidation is a potential strategy for reducing obesity and metabolic diseases [48]. The decrease in lipid content, as evidenced by Oil Red O staining of the mitochondria treated adipocytes compared to the untreated ones, is indicative of the potential to reverse or mitigate adipocyte hypertrophy. The mitochondria treated adipocytes released more free fatty acid and free glycerol, indicating enhanced lipolysis. To compare the basal lipolysis of the mitochondria treated adipocytes with stimulated lipolysis, a β3-adrenergic specific drug, CL316,243 was used. The lipolytic effects exerted by mitochondria therapy were like that of CL316,243. This finding was further substantiated by the mitotherapy-induced upregulation of the lipolysis enzyme genes such as Atgl, Lipe and CD36 which are responsible for breakdown of triglycerides leading to mobilization of excess lipid stores. Enhanced lipolysis leads to the utilization of lipids for energy production, and improved oxidation of fatty acids, thereby ameliorating lipid accumulation and its associated health risks.

The alteration in lipid content coupled with the intriguing finding that exogenous mitochondria transfer led to significant suppression of the adipocyte maturation genes like Adipoq, Adipisin and Ppar, provides support to the hypothesis that mitochondrial transfer may be able to modulate adipocyte differentiation process. Collectively, these findings underscore the therapeutic potential of exogenous mitochondria transfer in addressing key aspects of obesity-associated metabolic dysregulation.

The results of the cytokine expression levels revealed some unexpected findings. This study questioned the generally accepted view that exogenous mitochondria transfer reduces IL-6 levels. While previous research had shown a reduction in IL-6 levels in the context of injury models like acute kidney and neurological injuries, this study unveiled a contrasting phenomenon [49, 50]. It has been demonstrated for the first time that exogenous mitochondria transfer can increase IL-6 levels in adipocytes in vitro. This discovery holds particular significance because of the extensively studied role of IL-6 in adipose tissue metabolism and obesity.

The impact of IL-6 is well-documented, even within the context of interventions like CL316,243, a beta-3 adrenergic agonist, where IL-6 has been identified as a key mediator of its effects [18, 49, 50]. This finding thus emphasizes the role of IL-6 in the metabolic processes associated with obesity and, by extension, in the potential mechanisms behind mitochondria therapy.
To investigate the hypothesis that IL-6 plays a role in mitochondria therapy, a set of experiments were subsequently conducted. These experiments aimed to determine whether neutralizing IL-6 would yield different outcomes in the mitochondria therapy-induced enhancements in adipocyte energy expenditure, oxidative capacity, and lipid metabolism. The results were indeed striking. Neutralizing IL-6 using a monoclonal antibody led to a significant decrease in the expression of several key genes: *Ucp1*, *Dio2*, *Cox7a1*, *Ppargc1a*, and *Ppara*. This reduction was consistent with the previously established role of IL-6 in the browning of white adipose tissue [51].

Consequently, it was affirmed that IL-6 indeed plays a significant role in the mechanisms underlying mitochondria therapy. An interesting observation was the acute nature of the increase in IL-6 levels, which peaked shortly after treatment, yet returned to baseline levels by the 72-hour mark. However, gene expression of IL-6 remained elevated even after 72 hrs. This prompts questions regarding whether the transient IL-6 increase might operate similarly to the acute surge in IL-6 levels induced by physical exercise [52, 53].

The findings from this study suggest that the effects mediated by mitochondria therapy must be at least partially dependent on IL-6. These cumulative findings shed light on the specific mechanisms by which exogenous mitochondrial transfer exerts its therapeutic effects. It suggests that introducing exogenous mitochondria into metabolically compromised cells can stimulate multiple signaling pathways. This is particularly intriguing since exogenous mitochondria are not only foreign to the recipient cells but also possess the functional capacity to induce metabolic changes. Consequently, while the efficacy of mitochondrial transfer is actively studied, this study emphasizes the vital role of understanding the mechanisms that support its clinical safety and efficacy.

**Limitations of the Study**

Although this study's results provide insightful information about the interactions among adipocyte metabolism, interleukin-6 (IL-6), and exogenous mitochondrial transfer, it is important to recognize the limitations that may impact the interpretation and applicability of the findings.

Foremost, the primary methodology employed in this study was the utilization of an in vitro model with NIH3T3-L1 adipocytes. While in vitro models offer a controlled setting for studying biological pathways, they fail to fully replicate the complexity of *in vivo* systems. The use of a single cell line of murine origin may fail to account for the heterogeneity in human adipose tissue.

With a treatment duration of 72 hrs, the study focused mostly on short-term effects. It is important to consider that the long-term implications of exogenous mitochondrial transfer may differ from the observed short-term responses, particularly in the context of obesity and metabolic dysregulation. To alleviate this shortcoming, longitudinal research or longer treatment durations will be needed.
The findings suggest that there was a rise in IL-6 levels after mitochondrial therapy. Nevertheless, the specific processes via which mitochondria impact the synthesis of IL-6 have yet to be completely elucidated. Investigating the signaling pathways and regulatory mechanisms implicated in the release of IL-6 in response to mitochondrial transfer might yield a more comprehensive understanding of this interaction.

Another limitation of this study is the absence of an investigation into the combined treatment of exogenous mitochondria and IL-6. This raises questions on the potential synergistic or antagonistic effects of the interaction between exogenous mitochondria and IL-6. A dual-treatment strategy could have improved our understanding of how these factors interact to impact adipocyte metabolism in the context of obesity.

**Future Directions**

Building upon the findings and limitations of this study, several promising directions for future research emerge. These future directions aim to further elucidate the complex interactions between exogenous mitochondrial transfer, IL-6, and adipocyte metabolism in the context of obesity-associated dysregulation. The following research directions may provide valuable insights for advancing our understanding and potentially translating these discoveries into clinical applications:

1. Mechanistic Elucidation of Mitochondrial Transfer: The investigation of the exact processes behind the transfer of foreign mitochondria to host adipocytes is of utmost importance in order to gain a comprehensive understanding of this process. Gaining a comprehensive understanding of the mechanisms underlying the internalization of exogenous mitochondria, their interactions with the host's mitochondria, and their impact on cellular processes will yield valuable insights into this intricate process. Advanced microscopy techniques and molecular studies can shed light on the dynamics of mitochondrial transfer.

2. *In Vivo* Studies and Animal Models: To bridge the gap between in vitro findings and clinical relevance, *in vivo* studies using animal models are essential. Animal models, such as rodents, allow for the exploration of the long-term effects of exogenous mitochondrial transfer and IL-6 modulation. These models can provide insights into the safety and efficacy of potential therapeutic interventions in the context of obesity.

3. Cellular Plasticity: The observed downregulation of adipocyte maturation genes in response to exogenous mitochondrial transfer shows that adipocytes may be capable of cellular plasticity. Future research should look at the mechanisms behind this phenomenon and the reversibility of adipocyte differentiation. Understanding the extent of adipocyte plasticity in response to mitochondria therapy and its ramifications is an important area of study.

4. Effects of IL-6 Modulation: IL-6 is a pleiotropic cytokine that plays a variety of roles
in physiological processes. It is critical to investigate the potential off-target effects of IL-6 modulation to evaluate the safety and specificity of therapies. Future studies should look at the broader immunological and inflammatory effects of IL-6 modulation in context of exogenous mitochondria therapy.

5. **Longitudinal Investigations:** To understand the sustained impact of exogenous mitochondrial transfer, longitudinal studies are necessary. These studies should be conducted for longer treatment durations, monitoring metabolic changes over weeks or months, and examining the durability of the observed effects. Longitudinal data can provide insights into the feasibility of sustained interventions, an important factor in clinical application.

6. **Clinical Relevance and Human Studies:** Extending the research to human subjects is a critical step towards clinical translation. Clinical studies can investigate the effects of exogenous mitochondrial transfer and IL-6 modulation on human adipose tissue and metabolism. These studies should consider safety, efficacy, and the potential for personalized therapeutic approaches.

In conclusion, the future directions outlined above aim to address the limitations of this study and expand our understanding of the complex associations between exogenous mitochondrial transfer, IL-6, and adipocyte metabolism in the context of obesity-associated dysregulation. These research avenues possess the potential to make valuable contributions towards the development of novel strategies for the management of obesity and its associated metabolic effects.


APPENDIX. EXOGENOUS MITOCHONDRIA TRANSFER: A PROMISING THERAPEUTIC APPROACH FOR TREATING OBESITY

NOTE: Navigation with Adobe Acrobat Reader or Adobe Acrobat Professional: To return to the last viewed page, use key commands Alt/Control+Left Arrow on PC or Command+Left Arrow on Mac. For “Next view,” use Alt/Control+Right Arrow on PC or Command+Right Arrow on Mac. See Preface for further details. If needed, use this link to return to Chapter 2 after navigating within this appendix.

Introduction

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Manuscript

Exogenous Mitochondria Transfer: A Promising Therapeutic Approach for Treating Obesity

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Abstract

Obesity is a global health concern associated with various metabolic complications, including insulin resistance, dyslipidemia, and inflammation. Mitochondrial dysfunction has emerged as a significant component in the pathophysiology of obesity-related metabolic diseases. In recent years, exogenous mitochondrial transfer has garnered significant attention as a potential therapeutic approach for obesity. This review aims to explore the current understanding of mitochondrial dysfunction in obesity and evaluate the emerging evidence supporting the use of exogenous mitochondria transfer as a novel therapeutic strategy. We discuss the putative processes that underpin the beneficial effects of exogenous mitochondria transplantation in mitigating obesity-related metabolic abnormalities and highlight the challenges and future directions in this exciting field.

Introduction

1.1 Obesity: An Overview

Obesity and overweight diagnoses apply to over 40% of American adults as of 2020 (CDC). Additionally, one in five children are estimated to carry the diagnosis of obesity (CDC) and will typically go on to have the diagnosis in adulthood. Obesity in adults is defined as a body mass index of greater than 30kg/m². Though a flawed measurement system, it remains the most accessible and cheapest modality for screening purposes to identify patients with overweight or obesity, and it is a universal system of measurement.

In recent years, there has been a strong push to change the way of thinking about obesity as a need to regulate one’s diet and exercise to a true disease process that benefits from having medical assistance either by medications or surgical procedures. Currently, there are several mechanisms for weight loss recommended and/or approved in the United States. First, and to no one’s surprise, is lifestyle modifications. These include changes in diet and increased physical activity, though they also include treatments
through behavioral counseling and motivational interviewing [1]. More recently, medications have been encouraged as a means of weight loss. The current FDA approved medications include semaglutide, liraglutide, naltrexone/bupropion, phentermine/topiramate, and orlistat (NIDDK, 2023). The most invasive form of weight loss currently in use is bariatric intervention or surgery. These are all valid and recommended forms of obesity management, though each comes with its drawbacks and limitations.

Obesity can present with associated complications of hypertension, insulin resistance, diabetes, fatty liver, and metabolic syndrome. Altogether, this costs an estimated 173 billion dollars annually in treatment and care for these patients (CDC). Given the seriousness of complications, large cohort of patients affected, and economic costs, several treatment options should be researched and explored for benefit, as it is likely that not all patients will respond to the current therapies available. Therefore, we present this manuscript to discuss another possible means for therapy.

1.2 Mitochondrial Dysfunction in Obesity.

While excessive calorie intake and sedentary lifestyles are recognized as primary factors contributing to obesity, emerging data suggests that mitochondrial dysfunction plays a crucial role in the development and progression of obesity-related complications. Mitochondria, often attributed to be the "powerhouses" of the cell, are responsible for cellular energy production and various metabolic processes. In obesity, dysfunctional mitochondria contribute to impaired energy metabolism, oxidative stress, and altered cellular signaling. Several studies have demonstrated the presence of mitochondrial dysfunction in obesity [2].

Obesity is characterized by chronic low-grade inflammation, which plays a pivotal role in the development of related metabolic complications. Adipose tissue, particularly visceral adipose tissue, is a major source of pro-inflammatory cytokines and chemokines that contribute to the inflammatory state observed in obesity. Also, mitochondrial oxidative stress observed in obesity has significant implications for cellular function and metabolic health. The excessive production of ROS can damage mitochondrial DNA, proteins, and lipids, impairing their function [3]. Mitochondrial DNA mutations and deletions have been observed in adipose tissue of obese individuals [4]. These mitochondrial impairments contribute to the development of insulin resistance, dyslipidemia, and other metabolic abnormalities associated with obesity. The mitochondria population in cells is regulated by a process called mitophagy which involves specific phagocytosis of damaged mitochondria [5]. Mitophagy is responsible for maintaining a stable mitochondrial quality control system for cell function, such that there is an appropriate balance of energy production versus oxidative stress, and
dysregulated cells are repaired or disposed of. It has been shown that diet induced obesity impairs mitophagy by interfering with a cardiolipin mediated pathway [6]. Notably, studies have also linked diet induced obesity to reduced mitochondrial biogenesis and altered mitochondrial dynamics [7, 8]. Thus, targeting mitochondrial dysfunction through various therapeutic strategies presents a promising approach to manage obesity-related complications and improve overall metabolic health.

1.3 Obesity associated complications.

Hepatic Steatosis: Hepatic steatosis is a major comorbidity of obesity that is having an increasingly high impact on morbidity and mortality in the United States. Nonalcoholic fatty liver disease is on the rise and currently, without medical treatment, a growing cause for requiring liver transplant in patients [9]. Beaulant et al determined that a high-fat diet in mice lead to decreased mitochondria contact sites with the endoplasmic reticulum for calcium transport [10] When these contacts are absent, calcium concentration increases in the ER, causing ER stress, and mitochondrial oxidation decreases without available calcium to perform its functions [11]. These sites of ER-mitochondria interaction also assist with activation of PKB, in response to insulin presence [12]. When the ER is stressed, c-Jun N-terminal kinase is hyperactivated, which phosphorylates insulin receptor substrate 1, inhibiting the effects of insulin [13] The effective absence of insulin in the liver leads to glycogenolysis and gluconeogenesis [14]. Excess glucose in the liver is then transitioned towards fatty acid synthesis and ultimately into triglycerides [15]. IL-1β, which circulates peripherally due to the chronic inflammatory state caused by obesity and high fat diets, increases fatty acid synthesis in the liver, which then accumulates in hepatocytes [16].

Hypertension: increased caloric intake has been shown to have a causal relationship with sympathetic nerve activity [17]. The sympathetic nervous system in the kidneys are known to cause sodium reabsorption, ultimately leading to the same effect as the renin-angiotensin-aldosterone system pathway [18] Additionally, the hormone leptin, which is released proportionally compared to the amount of adipose tissue present, has been to act as a pressor by increasing sympathetic tone [19]. Obesity has also been proven to cause glomerular hyperfiltration, which may also be implicated in obesity-induced hypertension [20].

Coronary artery disease (CAD) and heart failure: Obesity causes a prolonged state of inflammation, with release of cytokines such as IL-6 and TNF-α. Coronary artery exposure to these cytokines leads to a long pathway that ultimately results in release of TGF-β which leads to collagen deposition into the myocardium and induces
cardiomyocyte hypertrophy [21]. Additionally, stress on the vascular endothelium in high blood pressure, also damages the endothelium leading to an increase in the pro-inflammatory cascade in the coronary arteries [22]

End stage renal disease (ESRD): As above, obesity has been found to cause hyperfiltration of blood in the kidney. This would be associated with increased intraglomerular pressure and cause damage to the glomerulus and nephron [23] Without intervention in the RAAS, this cycle of hyperfiltration leading to kidney damage goes unchecked resulting in a vicious cycle that perpetuates kidney damage.

2. Obesity and Mitochondrial Dysfunction

2.1 Role of adipose tissue mitochondria

Obesity is characterized by widespread expansion of white adipose tissue. The most important roles of white adipose tissue are systemic energy homeostasis and lipid metabolism. In nutrient excess state, white adipose tissue serves as a “reservoir” for storage of excess fat as triglycerides. Mitochondrial enzymes such as acetyl CoA carboxylase and fatty acid synthase play key roles in fatty acid synthesis and triglyceride production [24]. Additionally, in nutrient deficient state, mitochondria in white adipose tissue facilitates the lipolysis process, that is, breakdown of triglycerides into fatty acids and glycerol which are used by other tissues for their metabolism. Triglyceride breakdown is an enzymatic process which requires ATP provided by oxidative phosphorylation in mitochondria. Mitochondria in white adipose tissue play are critical for efficient energy and lipid homeostasis [25].

The adipose tissue has also been shown to have important endocrine functions that regulate functions of other tissues in the body [26]. They are involved in secretion of hormones (adiponectin, leptin, resistin, visfatin etc) and signaling molecules (ceramides, diacylglycerol, adipsin etc) collectively known as adipokines, which have crucial roles in maintaining insulin sensitivity and anti-inflammatory responses. The secretion of these adipokines is also regulated by mitochondria and hence, mitochondrial dysfunction can possibly lead to insulin resistance and impairment of anti-inflammatory responses [27].

White adipocytes are large, rounded cells with a single unilocular lipid droplet which occupies almost 90% volume of the cell, which results in the flattened nucleus being located in the periphery and low number of mitochondria in the white adipocytes [28, 29]. In contrast, brown adipocytes higher number of mitochondria which are spherical and packed with cristae, with abundant expression of UCP1 which is mediates generation of heat through non-shivering thermogenesis, specifically in infants and children, but has a role in adult thermogenesis as well.[30-32]
Characterization of mitochondrial protein by Forner et al demonstrated that oxidative phosphorylation, fatty acid metabolism, and the citrate cycle were predominant in BAT, while the mitochondrial proteins characterized in WAT were more consistent with rates of glycerolipid and fatty acid biosynthesis [33].

In obese individuals, glucose uptake by brown adipose tissue is decreased, predominantly due to a decreased density of capillaries in BAT, similar to the rarefaction of capillaries in WAT [34]. This has been corroborated by studies showing that a depletion of vascular endothelial growth factor A lead to whitening of BAT[35], while an abundance of VEGFA lead to browning in WAT [36].

2.2 Mechanisms of Mitochondrial Dysfunction in Obesity

The association of mitochondrial dysfunction and obesity has been established through various studies. The impaired mitochondrial activity is responsible for development and exacerbation of several complications associated with obesity [37]. Some of the major factors contributing to mitochondrial dysfunction in obesity are as follows.

2.2.1. Metabolic overload and lipotoxicity.

In obesity, there is excessive nutrients in the body which causes an increased entry of fatty acids and glucose in adipose tissue which causes a substrate overload in the mitochondria. This nutrient overload disrupts several essential mitochondrial processes such as ATP production, oxidative phosphorylation and results in increased ROS production [38].

Excessive lipid accumulation in adipose tissues as well as ectopic sites such as skeletal muscle and liver, is a characteristic of obesity. The accumulation of lipids, particularly saturated fatty acids, can induce lipotoxicity, leading to mitochondrial dysfunction. Saturated fatty acids can disrupt mitochondrial membrane integrity, impair oxidative phosphorylation, and increase ROS production, contributing to mitochondrial dysfunction and insulin resistance [2]. Giebelstein et al demonstrated that, in states of insulin resistance, skeletal muscle cells show increased glycolytic, but decreased TCA cycle and mitochondrial transport protein levels [39]. This could theoretically lead to an overload of mitochondrial substrate and favoring of free fatty acid oxidation and lipolysis for energy stores [40].

2.2.2. Oxidative stress and inflammation.

Mitochondrial oxidative stress is a huge contributor to mitochondrial dysfunction in obesity. Over production of reactive oxygen species (ROS) occurs within the
mitochondria which arises from dysfunctional electron transport chain [41]. When a high fat diet is ingested and processed by the mitochondria, genes involved in oxidative phosphorylation are downregulated. This disruption is caused by the excess nutrient state in obesity that leads to overdrive of the mitochondrial substrate oxidation and downregulation of genes involved in oxidative phosphorylation, limiting generation of new components of the electron transport chain [42]. This impairs the normal activity of the electron transport chain complexes resulting in disturbance in the normal flow of electrons and causes leakage of electrons to molecular oxygen. This results in mitochondria that are unable to fully restore their NADH and FADH$_2$ back to NAD$^+$ and FAD$^+$, which leads to generation of superoxide radicals contributing to oxidative stress. which leads to excess reactive oxygen species. Additionally, when there is excess nutrient intake, a decrease in expression levels of OXPHOS complexes I, II, III and IV as well as loss of mitochondrial membrane potential were noted in the subcutaneous adipose tissue of obese patients, when compared against controls [43]. Similarly, another study showed that there was a significant decrease in the protein levels of OXPHOS proteins complex III, IV and V in obese twins when compared to lean twins [44]. However, Fischer et al. pointed out that complexes I and IV are hit the hardest when compared between obese and non-obese women [45]. Furthermore, key antioxidant enzymes such as superoxide dismutase and catalase which help in maintaining redox balance are found to be deficient in obesity leading to oxidative damage [46].

Additionally, in obesity there is decrease in the levels of non-enzymatic antioxidants such as glutathione (GSH) and ROS scavengers vitamins C and E which are crucial to maintaining the redox balance thereby further weakening the antioxidant defense system [47, 48]. The increased ROS levels in obesity reduce mitochondrial ATP production and glutathione, and cause damage to the mitochondrial DNA, lipid and proteins [49].

Oxidative stress is further amplified by the chronic low-grade inflammation associated with obesity. Excess nutrients in obesity induces hypertrophy of the adipocytes which brings about a hypoxic state in the expanding tissue[50]. A high fat diet was found to increase the amount of hypoxia-inducible factor-1 alpha (HIF1α) in adipose tissue after 12 weeks, suggesting that oxygenation is inversely correlated to the amount of adipose tissue [51]. Additionally, the studied hypoxic tissues were found to have higher concentrations of M1-like proinflammatory macrophages, with a number that increased as the 12 weeks of the diet progressed. This hypoxic state induces activation and infiltration of the M1-like macrophages into adipose tissue that give rise to over production of cytokines such as IL-6, IL1β, TNFα as well as chemokines such as MCP-1, adiponectin and resistin [28, 52]. These pro-inflammatory cytokines and chemokines exacerbate the oxidative stress[41].
2.2.3 **Impaired mitochondrial biogenesis and dynamics.**

Obesity is associated with impaired mitochondrial biogenesis, the process of generating new mitochondria. Mitochondrial biogenesis and mitophagy work concurrently to maintain mitochondrial dynamics [53]. The key regulator of mitochondrial biogenesis is peroxisome proliferator-activated receptor gamma coactivator 1 alpha (Ppargc1a) which exerts its effects by induction of transcription factors such as Nuclear respiratory factor 1 (NRF-1), NRF-2, and mitochondrial transcription factor A (mtTFA). These transcription factors are essential for activation of mitochondrial genes and replication of mtDNA. [54, 55]. Reduced expression of Ppargc1a, such as (has been observed in obesity [56, 57]. Additionally, obesity alters mitochondrial dynamics, with increased mitochondrial fission and decreased fusion [7]. Altered mitochondrial dynamics disrupts mitochondrial quality control mechanisms, impairing the removal of damaged mitochondria through mitophagy [58].

2.2.4 **Insulin resistance.**

Insulin resistance is a key pathological feature of obesity and a major contributor to the development of type 2 diabetes mellitus. It is defined as the condition in which cells in different organs cease to respond adequately to the circulating insulin. In obesity, due to nutrient excess there are higher levels of circulating free fatty acids which tend to get deposited in non-adipose tissue such as heart, liver, and skeletal muscles as triglycerides. This impairs glucose and lipid metabolism aided by insulin signaling, thereby causing insulin resistance in the adipose tissue as well as the non-adipose tissues in other organs [59].

Growing evidence suggests that mitochondrial dysfunction plays a very important role in the pathogenesis of insulin resistance associated with obesity. Impaired mitochondrial oxidative phosphorylation can lead to decreased adiponectin secretion, potentially contributing to insulin resistance and metabolic dysregulation [60]. Excessive ROS production impairs insulin signaling pathways, leading to insulin resistance [61]. Furthermore, in a study done by Wu et al, a genetic modification (knock-out FUNDC1) causing impaired mitophagy demonstrated increased deformed mitochondria and oxidative stress, by hyperactivation of MAPK signaling. MAPK activation leads to downstream blocking of insulin-signaling transduction, leading to insulin resistance [62]. The activation of stress kinases, such as c-Jun N-terminal kinase (JNK) and IκB kinase (IKK), increases the phosphorylation of the insulin signaling pathways, disrupting the interaction of insulin receptor with its substrate and effectively inhibiting insulin receptor substrate (IRS) signaling[63].

3.1 Principles of exogenous mitochondria therapy

Mitotherapy or exogenous mitochondria transfer/transplantation is an emerging therapeutic approach aimed at treating various diseases by targeting mitochondrial dysfunction. It involves the transfer or administration of healthy and functional mitochondria to cells or tissues with compromised mitochondrial function.

The concept of mitochondria transfer was first elucidated by Clark and Shay [64]. They were the first to isolate mitochondria using a protocol consisting of differential centrifugation and purification using a sucrose gradient and co-incubated it with recipient cells. They used the isolated mitochondria to confer chloramphenicol and efrapeptin antibiotic resistance which was encoded in the mitochondria to the previously antibiotic sensitive cells [64]. Subsequently, it was shown that cell to cell mitochondria transfer occurs which is capable of reinstating mitochondrial respiration and improving energy production [65, 66].

The principles of mitotherapy revolve around the idea that healthy mitochondria can replace or supplement dysfunctional mitochondria, thereby restoring normal mitochondrial function and improving cellular bioenergetics. This approach makes use of the unique properties of mitochondria, including their ability to undergo fusion and fission, their dynamic nature, and their capacity for intercellular communication [67].

3.2 Mechanism of exogenous mitochondrial uptake

A very important factor determining the success of mitotherapy is the ability of the exogenous mitochondria to integrate and function within the recipient cells. While there has not been a consensus yet regarding the precise mechanism through which this integration occurs, there are studies that have shed some light on the processes involved. One of the proposed mechanisms is uptake of exogenous mitochondria through endocytosis, where recipient cells engulf the transplanted mitochondria in membrane-bound vesicles [66, 68].

Another proposed mechanism is that of macropinocytosis which is a distinct endocytotic pathway that involves non-specific uptake of vast volumes of extracellular fluid, solutes, and membrane in big endocytic vesicles known as macropinosomes [69]. In 2014, a study by Kitani et al showed that co-incubation of human uterine endometrial epithelial cells with DsRed2-labelled mitochondria (also isolated from EMCs) and EIPA, an inhibitor of macropinocytosis, resulted in suppressed transfer, as confirmed through fluorescence microscopy and FACS analysis [70]. It was further verified by using other inhibitors of macropinocytosis and endocytosis, such as cytochalasin D (actin
polymerization inhibitor) and nocodazole (microtubule assembly inhibitor), which resulted in reduced mitochondrial transfer, while an inhibitor of clathrin-mediated endocytosis, chlorpromazine had no effect [70]. However, this was refuted by Pacak et al found that endocytosis of exogenous mitochondria is an actin dependent process as a conformational change in actin filaments in the cytoskeleton leads to development of invaginations of the plasma membrane that are required for endocytosis and can be inhibited by cytochalasin D [71].

Although tunneling nanotubes (TNTs) have been indicated as a mechanism in intercellular mitochondrial transfer [72, 73] it was not found to be applicable for exogenous isolated mitochondria transfer [71, 74]. In a 2016 study, Kesner et al upheld that macropinocytosis was indeed the mechanism for internalization of exogenous mitochondria by showing that only inhibitor of macropinocytosis and not that of clathrin mediated endocytosis prevented mitochondrial transformation [75]. Furthermore, they also found that the integrity of the mitochondrial outer membrane was crucial for successful uptake of the exogenous mitochondria [75]. However, it is worth noting that studies by Pacak et al and Kesner et al were performed using different types of cells which could imply that there may be contribution of more than one type of mitochondrial transfer mechanism at play [71,74].

3.3 Rationale for Utilizing Exogenous Mitochondria Transfer in Obesity

Owing to the importance of proper mitochondrial function in maintenance of energy and metabolic homeostasis in adipose tissue, transfer of isolated healthy mitochondria into adipose tissue may be a promising therapeutic strategy to combat obesity. Recently it has come to light that intercellular mitochondrial transfer occurs from white adipocytes to white adipose tissue (WAT) macrophages as a form of immunometabolic crosstalk [76]. This study showed that there was a decrease in the intercellular mitochondrial transfer from adipocytes to macrophages in diet induced obesity using chow fed versus high fat diet fed mice. This was found to be caused by a deficiency in the heparan sulfate in the surface of the WAT macrophages which impaired its ability to uptake the mitochondria. Furthermore, in a 2022 study by the same group, it was found that dietary long chain fatty acids (present in a western high fat diet) play a role in preventing the intercellular transfer of mitochondria from white adipocytes to macrophages and redirecting the cell free mitochondria to the blood and subsequently to other organs for regulating nutrient stress induced metabolic adaptation [77].

Recently, there have been several preclinical studies that exploited the therapeutic benefit provided by mitochondria transfer in the context of metabolic disorders. Notably, in 2017, Fu et al. demonstrated that treatment of high fat diet induced fatty liver mice with exogenous mitochondria, isolated from human hepatoma cells, were successful in
ameliorating the excess lipid accumulation and oxidative stress in the mice livers as well restored energy production and normal hepatocyte function [78]. While this is one of the rare studies which employed isolated exogenous mitochondria as therapeutic material, many of the studies conducted have employed mesenchymal stem cells mediated mitochondrial transfer. In this context, a study by Bi et al. in 2021 showed that bone marrow derived mesenchymal stem cell derived mitochondria treatment in a murine Type 2 DM associated NAFLD model rescued hepatocyte function by reducing steatosis, improving OXPHOS activity and ATP production of the cells. A significant finding of this study was that inhibition of the mitochondria transfer suppressed the therapeutic effects of the BMSC transfer. This proved that it was not simply the paracrine signaling of the BMSCs, rather the event of mitochondrial transfer which contributed as a dominant factor in the alleviation of steatosis of the hepatocytes [79]. Yet another study showed that mesenchymal stromal cells improved insulin secretion by both human and mice islet beta cells in vitro, by virtue of mitochondria transfer mediated improvements [80, 81]. These studies provide sufficient evidence that exogenous mitochondria transfer can become an effective therapeutic technique to treat metabolic disorders such as obesity and associated complications such as hepatic steatosis, insulin resistance and cardiomyopathy among others.

There are several ways through which mitotherapy alleviates mitochondrial dysfunction. Studies have shown that replacement of damaged mitochondria with healthy exogenous mitochondria can boost ATP production by enhancing oxidative phosphorylation [82, 83]. These aid in restoration of energy production in cells with impaired mitochondrial function. Transferred mitochondria can also improve cellular bioenergetics by inhibiting the generation of excess reactive oxygen species (ROS) and regulating redox balance [84].

Furthermore, transplantation of exogenous mitochondria can modulate the Ppargc1a pathway, possibly by activating its upstream pathways such as p38 MAPK, Calcineurin A and CaMK or AMP activated protein kinase (AMPK) pathway [85-88]. Activation of these pathways enhances mitochondrial biogenesis which results in the formation of healthy mitochondria, thereby increasing the number of functional mitochondria [89, 90].

4. Exogenous Mitochondria Therapy in Preclinical and Clinical Models

4.1 Source of Exogenous Mitochondria

The success of mitotherapy relies on selecting appropriate sources of exogenous mitochondria. These sources may be either cell derived or healthy tissues. Autologous, allogeneic, and donor mitochondria, as well as mitochondrial donation, are potential sources for this technique. Each source has its advantages and limitations, which must be
carefully considered based on the specific context and goals of mitochondrial transplantation.

Preclinical studies using in vitro and in vivo methods have used mitochondria isolated from several cell lines. One of the prominent cell types used for this purpose is mesenchymal stem cells (MSCs) which may derive from different origins such as adipose tissue, bone marrow, umbilical cord among others [84, 91]. Transfer of isolated mitochondria have been shown to be effective in treating acute ischemia reperfusion injury in brain and heart as well as in models of acute lung injury and musculoskeletal disorders [73, 92-95]. A recent study has shown that mitochondria isolated from human bone marrow-MSCs had better cardioprotective effects in a murine myocardial infarction model when compared to mitochondria isolated from a human fibroblast cell line HFF-1 [96]. As seen from the studies discussed above, mitotherapy using mesenchymal stem cells derived from mitochondria has been especially favored in case of various models of metabolic disorder. An interesting study conducted in 2019 by Konari et al showed that isolated mitochondria from bone derived mesenchymal stem cells was capable of improving oxidative stress, mitochondrial biogenesis and restoration of structure of renal tubules damaged by streptozocin induced diabetic nephropathy in rats when injected directly under the renal capsule [97].

Other cell lines such as HepG2, HeLa, PC12 have also been successfully used as the source of mitochondria as demonstrated in several recent studies [98-100]. A major advantage of using cultured cells to isolate mitochondria is that it can be done without inflicting any additional distress to the recipient in contrast to autologous mitochondria transfer. Furthermore, there is no heterogeneity in the cultured cell population causing variables in the treatment. Mitochondria isolated from tissues such as liver and skeletal muscles have been used to inject intravenously in murine models of ischemia reperfusion and carbon tetrachloride induced liver injury, heart ischemia reperfusion injury and muscle injury respectively [74, 101-103].

While autologous sources eliminate the risk of immune rejection and can be isolated from different cell types and tissues of the recipient, there may be limited quantity and compromised quality of the mitochondria in cases of patients who have multiorgan mitochondrial dysfunction. Another drawback is it would further inflict damage and distress to the recipient to obtain their tissues to isolate mitochondria from. However, a drawback is that it is more time consuming and may not be a feasible option when a bulk number of mitochondria is needed for injections in clinical settings. Allogeneic mitochondria derived from various tissues such as liver, heart and skeletal muscle would be obtained from individuals genetically different from the recipient. Hence the potential risk of immune rejection and compatibility issues must be considered when using allogeneic sources.
Donor mitochondria obtained from organ donors or isolated from cell lines can offer a diverse pool of healthy mitochondria for the purpose of mitotherapy in clinical settings. Donor mitochondria can be derived from various tissues and cell types, including skeletal muscle, placenta, and umbilical cord blood [104].

Mitochondrial donation, involving transfer of healthy mitochondria from a donor individual to a recipient with mitochondrial dysfunction has been explored in cases of mitochondrial DNA (mtDNA) mutations, particularly in assisted reproductive technologies [105]. However, ethical considerations, availability, and the potential risk of immune reactions need to be carefully evaluated when using donor mitochondria [106].

4.2 Methods of Delivery

The efficacy of this technique relies not only on the quality and viability of the exogenous mitochondria but also on the delivery methods employed. Different delivery methods can significantly influence the outcomes of mitochondrial transplantation by affecting the efficiency of mitochondrial transfer, cellular uptake, and subsequent functional integration. Delivery may be targeted such as in direct injection or systemic, as in the case of intravenous/intra-arterial injection and even via nasal administration [100, 107]. Direct injection involves the localized delivery of exogenous mitochondria into target tissues or organs. This method allows for precise targeting and immediate access to the site of action. Studies have demonstrated the successful restoration of mitochondrial function and amelioration of tissue damage through direct injection in various disease models, including cardiac ischemia and Schizophrenia [92, 108].

Intravenous injections non-invasive approach allows for widespread distribution of mitochondria to multiple organs and tissues. Intravenous administration has shown promising results in improving mitochondrial function and attenuating organ injury in conditions such as fatty liver and CCL4-induced acute liver injury [78].

In context of obesity and associated metabolic disorders such as fatty liver and insulin resistance, each method has its advantages and limitations, which should be carefully considered according to treatment objectives. Direct injection allows for targeted delivery but may have limited diffusion and chances of tissue damage at site of injection, while intravenous administration enables systemic distribution but requires efficient cellular uptake. Hence, it is imperative that careful consideration be given to method of delivery in order improve targeting specificity and ensure the long-term functional integration of exogenous mitochondria.
4.3 Safety and Efficacy of Mitotherapy in Clinical Setting

In 2017, McCully and group were the first to demonstrate the use of autologous exogenous mitochondria therapy in human patients [109]. They used mitochondria isolated from rectus abdominis muscle from the pediatric patients suffering from cardiac ischemia reperfusion injury and then transferred the exogenous mitochondria directly into the hearts via epicardial injection. The results showed that there was improvement in the contractile function of the ventricular myocardium which enabled four out of five patients to be relieved from ECMO support. While this pioneering study is the first of its kind to show that mitotherapy is a viable therapeutic option, there is a need for development of alternate less invasive delivery methods. Furthermore, it is of paramount importance to ensure genetic and immunological compatibility between the donor mitochondria and recipient cells.

Ethical considerations also play a significant role in selecting a donor for exogenous mitochondria transplantation. The source of the donor mitochondria should be ethically acceptable, ensuring compliance with relevant regulations and guidelines. Safety considerations, such as screening for potential infectious agents or other contaminants, should also be addressed to minimize any potential risks associated with the transplantation process.

Conclusion

Mitochondrial transplantation has tremendous therapeutic potential in the treatment of obesity and related metabolic diseases. This technique offers a promising avenue for alleviating metabolic dysfunction by restoring mitochondrial activity and enhancing cellular energy metabolism. Transferring healthy exogenous mitochondria can repair damaged mitochondrial function, increase insulin sensitivity, and improve cellular energy metabolism. Stem cell-derived mitochondria and gene editing technologies may provide novel ways to improve the therapeutic efficacy and safety of mitochondrial transfer. Additionally, recently investigated targeted delivery techniques, such as nanocarriers and tissue-specific targeting peptides, may improve the specificity and efficacy of mitochondria transfer. Furthermore, identifying specific mitochondrial biomarkers linked to metabolic dysfunction can help in the selection of optimal donor mitochondria and the development of personalized treatment strategies. However, factors like the stability of isolated mitochondria, long-term preservation, and the technicalities involved in bulk synthesis for therapeutic applications must be thoroughly researched. Another potential issue with employing mitotherapy to treat obesity is the risk of increased immunological reactivity and pro-inflammatory conditions. Therefore, studies evaluating the long-term therapeutic effects of exogenous mitochondria transfer in large animal models and clinical trials are critical for determining its safety, efficacy, and long-term benefits.


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VITA

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