STUDIES ON EARLY MITOCHONDRIAL RESPONSE OF SH-SY5Y CELLS TO NICOTINE

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This is to certify that the dissertation entitled **"STUDIES ON EARLY MITOCHONDRIAL RESPONSE OF SH-SY5Y CELLS TO NICOTINE"** submitted to the University of Kerala in partial fulfilment of the requirements for the award of degree of Master of Science in Biochemistry, is a record of original research work carried out by **NAKSHATHRA.R (Candidate code: 63824142006)** of the Department of Biochemistry, T.K.M. College of Arts and Science, Kollam, under the guidance and supervision of Dr. Viji Vijayan, Assistant Professor, Department of Biochemistry, University of Kerala, Karyavattom. It is also certified that no part of this work has been presented for the award of any other degree, diploma, or similar title at any other university.

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This is to certify that the thesis work entitled **"STUDIES ON EARLY MITOCHONDRIAL RESPONSE OF SH-SY5Y CELLS TO NICOTINE"** is a bonafied record of research work which has been carried out by **NAKSHATHRA R (Candidate code: 63824142006)** in partial fulfilment for the award of the Degree of Master of Science in Biochemistry, Department of Biochemistry, T.K.M. College of Arts and Science, Kollam under my supervision and guidance at the Department of Biochemistry, University of Kerala, Karyavattom, Thiruvananthapuram. The content of this dissertation, in full or in part, has not been submitted to any other Institute or University for the award of any degree or diploma.

Dr. Viji Vijayan
Supervising Guide

Thiruvananthapuram,
July 2026

DECLARATION

I, hereby declare that the project work entitled **"STUDIES ON EARLY MITOCHONDRIAL RESPONSE OF SH-SY5Y CELLS TO NICOTINE"** submitted to the University of Kerala, is a record of an original work done by me under the guidance of Dr. Viji Vijayan, Assistant Professor, Department of Biochemistry, University of Kerala, Thiruvananthapuram and this project work is submitted in the partial fulfilment of the requirements for the degree of Master of Science in Biochemistry. The results embodied in this thesis have not been submitted to any other University or Institute for the award of any degree or diploma.

NAKSHATHRA. R

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LIST OF ABBREVATIONS

AMPK: AMP-Activated Protein Kinase

APS: Ammonium Persulfate

ATP: Adenosine Tri Phosphate

BR: Broad Range

BSA: Bovine Serum Albumin

Ca^{2+}: Calcium Ions

CO_2: Carbon Dioxide

COX IV: Cytochrome c Oxidase Subunit IV

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic Acid

Drp1: Dynamin-Related Protein 1

ER: Estrogen Receptor

ERR: Estrogen-Related Receptor

FBS: Fetal Bovine Serum

FM: Furan-Maleimide

HAT: Histone Acetyltransferase

IMM: Inner Mitochondrial Membrane

IS: Intermembrane Space

Mfn1: Mitofusin-1

Mfn2: Mitofusin-2

mRNA: Meesenger Ribonucleic Acis

mtDNA: Mitochondrial DNA

nAChRs: Nicotinic Acetylcholine Receptors

NADH: Nicotinamide Adenine Dinucleotide + Hydrogen

NaOH: Sodium Hydroxidee

NIH: National Institutes of Health

NRF: Nuclear Respiratory Factor

OMM: Outer Mitochondrial Membrane

Opa1: Optic Atrophy 1

OXPHOS: Oxidative Phosphorylation

PBS: Phosphate-Buffered Saline

PD: Parkinson’s Disease

PGC-1α: Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 Alpha

pH: Potential of Hydrogen

RIA: Radioimmunoprecipitation Assay

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

rRNA: Ribosomal Ribonucleic Acid

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SIRT 1: Sirtuin 1

TBS: Tris Buffered Saline

TBST: Tris Buffered Saline with Tween 20

TCA: Tricarboxylic Acid

TEMED: N,N,N’,N’-Tetramethylethylenediamine

TFAM: Mitochondrial Transcription Factor A

TIM23: Translocase of the Inner Membrane 23

TOM 20: Translocase of Outer Mitochondrial Membrane 20

Tris-HCl: Tris Hydrochloride

tRNA: Tranfer Ribonucleic Acid

USA: United States of America

WHO: World Health Organisation

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ABSTRACT

Nicotine exposure has been associated with neuronal dysfunction, but its early effects on mitochondrial homeostasis remain incompletely understood. This study evaluated the impact of acute low-dose nicotine exposure on mitochondrial status in SH-SY5Y neuronal cells. Cells were treated with 0.1 μM nicotine for 4 hours, followed by assessment of mitochondrial fluorescence intensity using MitoTracker Green FM staining and analysis of PGC-1α protein expression by Western blotting. Nicotine-treated cells showed a modest reduction in mitochondrial fluorescence intensity compared with untreated controls, suggesting early alterations in mitochondrial content, integrity, or organization. Western blot analysis revealed a significant decrease in PGC-1α expression, indicating suppression of mitochondrial biogenesis-related signaling. Although the fluorescence change was mild, the marked reduction in PGC-1α suggests that nicotine can affect mitochondrial regulatory pathways even after short-term exposure. These findings provide preliminary evidence that acute nicotine exposure negatively influences neuronal mitochondrial health and may contribute to nicotine-induced neurotoxicity.

1. INTRODUCTION

Neurons are highly specialized cells that require continuous energy supply to maintain membrane potential, neurotransmission, calcium homeostasis, axonal transport, and synaptic activity (Akers et al., 2014; Ahmed et al., 2019; Pronina et al., 2022; Yao et al., 2022; Akay, 2023). Because of this high metabolic demand, neuronal cells are particularly dependent on mitochondrial function (Hoover et al., 2025). Mitochondria generate ATP through oxidative phosphorylation and also regulate calcium buffering, reactive oxygen species production, apoptotic signalling, and cellular stress responses (Llorente et al., 2022; Dittmann et al., 2025). Therefore, even early changes in any proteins that regulate mitochondrial function can affect neuronal survival and homeostasis.

Mitochondrial health is maintained through a balance between mitochondrial biogenesis, dynamics, and quality-control pathways (Holloszy, 2011). Mitochondrial biogenesis is the formation of new mitochondria and is regulated by coordinated communication between nuclear and mitochondrial genomes (LeBleu et al., 2014). One of the key regulators of this process is peroxisome proliferator-activated receptor gamma coactivator-1 alpha, commonly known as PGC-1α. PGC-1α activates transcription factors such as nuclear respiratory factor-1 and nuclear respiratory factor-2, which regulate mitochondrial transcription factor A and other genes required for mitochondrial DNA replication, transcription, and respiratory chain function (Cameron et al., 2016; Cameron et al., 2017; dos Santos et al., 2018). Thus, PGC-1α is widely considered a master regulator of mitochondrial biogenesis and oxidative metabolism.

Nicotine is the major bioactive compound present in tobacco and nicotine-containing products. It primarily acts through nicotinic acetylcholine receptors and can alter neuronal excitability, intracellular calcium levels, neurotransmitter release, and cellular metabolism (Sansone et al., 2023). In addition to receptor-mediated actions, nicotine has also been reported to directly influence mitochondrial function by affecting respiratory chain activity, oxygen consumption, and reactive oxygen species generation. These mitochondrial effects are important because disruption of mitochondrial bioenergetics may contribute to neuronal stress and neurotoxicity. Previous studies suggest that nicotine can modulate mitochondrial respiration by affecting Complex I of the electron transport chain and intracellular calcium (Malinska et al., 2019). However, the early effect of low-dose nicotine exposure on mitochondrial abundance and mitochondrial biogenesis-related signalling in neuronal cells requires further investigation

2. AIM AND OBJECTIVES

2.1 Hypothesis

The present work hypothesizes that acute nicotine exposure affects mitochondrial homeostasis in neuronal cells by reducing mitochondrial fluorescence intensity and suppressing PGC-1α-mediated mitochondrial biogenesis signalling.

2.2 Aim

By combining mitochondrial fluorescence analysis with PGC-1α protein expression, this study aimed to provide preliminary insight into the early mitochondrial response of neuronal cells to nicotine exposure.

2.3 Objectives

The objectives were:

1. To evaluate the effect of acute nicotine exposure on mitochondrial status in neuronal cells using MitoTracker Green FM staining.
2. To assess whether nicotine alters mitochondrial fluorescence intensity in neuronal cells after 4 h of exposure.
3. To investigate the effect of nicotine on the expression of PGC-1α, a key regulator of mitochondrial biogenesis, using Western blot analysis.
4. To compare mitochondrial changes in basal and nicotine-treated neuronal cells.
5. To understand whether short-term nicotine exposure affects early mitochondrial homeostasis and mitochondrial biogenesis-related signalling in neuronal cells.

3. REVIEW OF LITERATURE

3.1 Brain

The human brain is one of the most complex biological systems, containing over 100 billion neurons interconnected through numerous synapses (Stiles et al., 2010). It is composed of nervous tissue and regulates sensory perception, movement, emotions, language, communication, memory, cognition, and behaviour (Pessoa, 2010). Structurally, the brain is divided into three major regions: the cerebrum, cerebellum, and brainstem (Figure1).

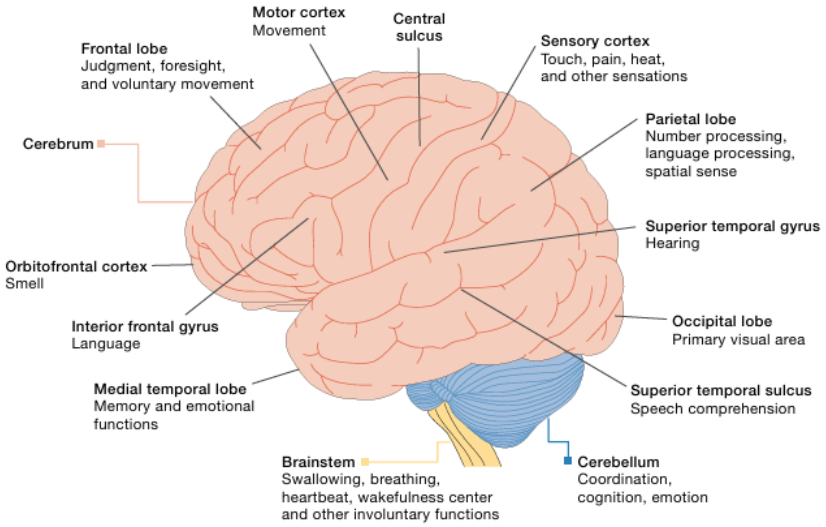


FIGURE 1: Regions of the brain. There are three principal sections of the brain: the cerebrum, cerebellum, and brainstem. Thirteen of the structural regions of cerebrum was shown along with some of their associated functions, though these regions are highly connected and can share responsibilities (Adapted from Thomas et al., 2020).

The cerebrum is the largest part of the brain and consists of right and left hemispheres. Its surface contains gyri, sulci, and fissures, and each hemisphere is composed of an outer gray matter layer called the cerebral cortex and underlying white matter. The cerebrum processes sensory and motor information and regulates conscious and unconscious behaviour, emotion, intelligence, and memory. The left hemisphere is mainly associated with speech and abstract reasoning, whereas the right hemisphere is involved in spatial reasoning. The cerebral cortex is divided into four lobes: frontal, parietal, temporal, and occipital (Sporns, 2011; Mochizuki et al., 2014; Sanders et al., 2016).

The cerebellum is located in the posterior cranial fossa and plays a major role in motor coordination, posture, and balance. It consists of the cerebellar cortex, which has molecular,

Purkinje, and granular layers, along with deep cerebellar nuclei. Through its connections with the brainstem, the cerebellum receives sensory information from the brain and spinal cord to refine voluntary movements. It also contributes to cognitive functions, including attention, language, pleasure response, and fear memory (Takakusaki et al., 2017; Manto, 2018; Jimsheleishvili et al., 2023; Unverdi et al., 2023).

The brainstem, composed of the midbrain, pons, and medulla, lies between the cerebrum and spinal cord, anterior to the cerebellum. It regulates vital autonomic functions such as respiration, heart rate, temperature regulation, sleep–wake cycles, digestion, swallowing, coughing, sneezing, and vomiting. It contains white matter tracts that transmit motor signals from the cerebral cortex and sensory information from the spinal cord and peripheral nerves to higher brain regions (Angeles Fernández-Gil et al., 2010; Hegarty et al., 2013; Singh and Vishram, 2014).

3.2 Cells of the brain

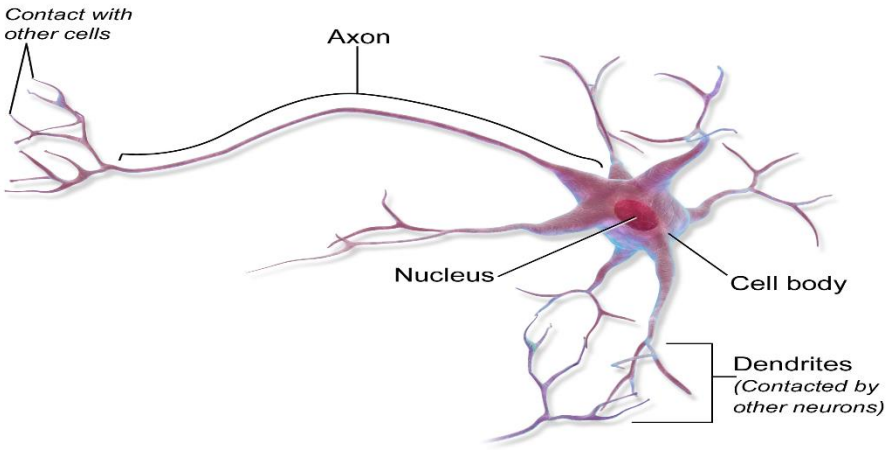


FIGURE 2: Neuron (Adapted from BruceBlaus, 2014)

Brain cells constitute the functional tissue of the brain and include diverse cellular populations such as neurons, glial cells, innate immune cells, and mesenchymal cells, each performing specialized roles in maintaining brain structure and function (Purves and Dale, 2012; Herculano-Houzel, 2009; Mason et al., 2016). Among these, neurons are the principal information-processing cells (Figure 2), whereas glial cells provide structural, metabolic, and functional support to the neuronal network. Glial cells help maintain the ionic environment required for neuronal excitability, regulate neurotransmitter uptake, modulate synaptic activity, support neural development, and participate in repair responses following injury. The major glial cell types include astrocytes, oligodendrocytes, and microglia (Herculano-Houzel, 2014;

Jakel et al., 2017; Mederos et al., 2019). Astrocytes are the most abundant glial cells in the brain and occupy nearly one-fourth of the total brain volume (Guillamón-Vivancos et al., 2015). These regulate cerebral blood flow, maintain the blood–brain barrier, provide metabolic support to neurons, and participate in tissue repair after infection or traumatic injury (Oberheim et al., 2012; Ransom et al., 2012; Clarke et al., 2013; Vasile et al., 2017). Astrocytes are broadly classified into protoplasmic astrocytes, found mainly in gray matter, and fibrous astrocytes, found mainly in white matter. Protoplasmic astrocytes have highly branched processes that interact with synapses and blood vessels, whereas fibrous astrocytes possess longer processes associated with nodes of Ranvier and vascular structures (Perez-Alvarez et al., 2013; Dallerac et al., 2016; Ghezali et al., 2016). Astrocytic end-feet also interact with endothelial cells and the basal lamina, contributing to blood–brain barrier formation and maintenance (Rajkowska et al., 2013; Santello et al., 2019).

Oligodendrocytes are the myelinating cells of the central nervous system. These form myelin sheaths around axons, which insulate nerve fibres, support rapid electrical impulse conduction, and improve conduction velocity (Butt and Rocha et al., 2019; Rivera et al., 2021; Butt and Papanikolaou et al., 2019; Vanzulli et al., 2020). Myelin sheaths are arranged in segments along axons and are separated by nodes of Ranvier, performing a function comparable to Schwann cells in the peripheral nervous system (Perry et al., 2013; Rivera et al., 2016).

Microglia are the resident immune cells of the central nervous system and play essential roles in immune surveillance, phagocytosis, synaptic remodelling, and response to injury or infection. Unlike neurons and macroglia, microglia do not arise from ectodermal tissue but originate from yolk sac progenitors that populate the brain during development (Kettenmann et al., 2011; Asai et al., 2015). These are distributed throughout the brain and continuously monitor the neural microenvironment. Microglia contribute to both innate and adaptive immune responses and protect the brain from pathological insults (Luo et al., 2010; Butovsky et al., 2018; Prinz et al., 2019; Zhang et al., 2022).

Neurons are the fundamental structural and functional units of the nervous system. These are specialized for receiving, processing, and transmitting information through electrical and chemical signals, thereby coordinating sensory perception, motor responses, cognition, and other essential physiological functions (Pronina et al., 2022; Yao et al., 2022; Akay, 2023). Neurons communicate with other neurons at synapses and with effector cells at neuroeffector junctions. A typical neuron consists of a cell body or soma, dendrites, and an axon (Figure 2).

The soma contains the nucleus and major organelles required for cellular metabolism and protein synthesis. Dendrites receive afferent signals from other neurons or the extracellular environment, whereas the axon conducts efferent signals away from the cell body towards target cells (Owusu et al., 2025; Ludwing et al., 2023).

Neurons exhibit marked structural diversity and are classified into multipolar, bipolar, pseudounipolar, and anaxonic types based on the number and arrangement of their axons and dendrites. Multipolar neurons, which possess one axon and multiple dendrites, are the most common neuronal type in the central nervous system. Bipolar neurons contain one axon and one dendrite and are commonly associated with special sensory pathways. Pseudounipolar neurons are typically involved in sensory transmission, whereas anaxonic neurons lack a clearly identifiable axon and are often involved in local circuit modulation. This structural diversity allows neurons to perform highly specialized functions across different regions of the nervous system.

The ability of neurons to transmit signals depends on the generation and propagation of action potentials. This process is driven by the movement of ions across the neuronal membrane through ion channels. Sodium, potassium, chloride, and calcium ions contribute to neuronal excitability and membrane potential, which typically rests around −70 mV (Akers et al., 2014; Ahmed et al., 2019; Llorente et al., 2022; Dittmann et al., 2025). When a neuron receives sufficient excitatory input, voltage-gated sodium channels open, leading to membrane depolarization and generation of an action potential. The electrical signal then propagates along the axon and reaches the axon terminal.

At the axon terminal, the electrical signal is converted into a chemical signal through the release of neurotransmitters, neuromodulators, or neurohormones into the synaptic cleft. These chemical messengers bind to receptors on the postsynaptic neuron or effector cell, allowing communication across synapses or neuromuscular junctions. Efficient neuronal function also depends on axonal transport, through which proteins, vesicles, organelles, and signalling molecules are transported between the soma and axon terminals. This transport is mediated by motor proteins such as kinesin and dynein, which move cargo along microtubules in anterograde and retrograde directions, respectively (Chen et al., 2020).

Since neurons are highly dependent on energy metabolism, calcium homeostasis, mitochondrial function, and synaptic integrity, these are particularly vulnerable to cellular

stress, oxidative damage, and mitochondrial dysfunction. In vitro neuronal models are therefore widely used to study mechanisms of neurotoxicity, neuroprotection, mitochondrial impairment, and synaptic dysfunction.

The SH-SY5Y cell line, derived from human neuroblastoma, is commonly used as a neuronal model because it exhibits several neuron-like properties and can be differentiated to express more mature neuronal characteristics (Targett et al., 2024). Therefore, SH-SY5Y cells provide a useful experimental system for investigating neuronal responses to pharmacological agents, toxic insults, oxidative stress, mitochondrial alterations, and neuroprotective interventions.

3.3 Mitochondria

Mitochondria are dynamic, multifunctional organelles present in nearly all human cells except mature red blood cells. It is a double membrane bound intracellular organelles having it's own genomes (Gray, 2012; Karnkowska et al., 2016). It has four distinct compartments: the outer mitochondrial membrane (OMM), the intermembrane space (IS), the inner mitochondrial membrane (IMM), and the mitochondrial matrix.

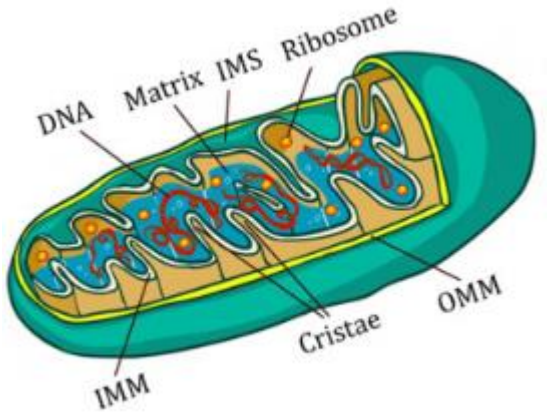


Figure 3: Structure of mitochondria (Adapted from Javadov et al., 2020)

Mitochondria are double-membrane organelles composed of an outer mitochondrial membrane (OMM), an inner mitochondrial membrane (IMM), an intermembrane space, and a matrix. The OMM is permeable to most ions and small molecules, whereas the IMM is highly selective and forms cristae that provide a large surface area for respiratory chain complexes and ATP synthase (Figure 3). Mitochondria possess their own DNA (mtDNA), which encodes 37 genes, including key subunits of the respiratory chain and mitochondrial rRNAs and tRNAs. However, most mitochondrial proteins are encoded by nuclear genes, synthesized in the

cytoplasm, and imported into mitochondria (Lee and Han, 2017). The mitochondrial matrix houses the tricarboxylic acid (TCA) cycle, whose intermediates support the synthesis of biomolecules such as lipids, amino acids, nucleotides, and heme (Adebayo et al., 2021; Moura et al., 2025).

Mitochondria are commonly referred to as the powerhouses of the cell because it generate ATP through oxidative phosphorylation (OXPHOS) (Friedman and Nunnari, 2014). In addition to energy production, they regulate calcium signalling, reactive oxygen species (ROS) generation, immune responses, and cell death pathways (Tait and Green, 2012; McCarron et al., 2013; Li et al., 2015). The OXPHOS system consists of electron transport chain complexes I–IV and ATP synthase, also known as complex V. Electron transfer through complexes I, III, and IV pumps protons across the IMM, generating an electrochemical gradient that drives ATP synthesis by complex V. The spatial organization of respiratory complexes along cristae and the concentration of ATP synthase at cristae ridges improve the efficiency of energy production (Nelson et al., 2008; Papa et al., 2012; Beaulant et al., 2022; Purandare et al., 2023).

Mitochondrial number and morphology vary depending on cell type, developmental stage, cell-cycle status, age, and environmental conditions (Lin, 2016; Wang and Zhang et al., 2017). Mitochondrial homeostasis is maintained through dynamic cycles of fusion and fission. Fusion allows exchange of mitochondrial contents and supports functional recovery during metabolic or environmental stress, whereas fission is required for mitochondrial division, quality control, and distribution within the cell (Adebayo et al., 2012; Adebayo et al., 2021; Gao and Wang et al., 2022). Besides ATP production, mitochondria also participate in the synthesis of ketone bodies, steroids, phospholipids such as cardiolipin and phosphatidylglycerol, amino acids including glutamate, glutamine, aspartate, asparagine, proline, and ornithine, as well as nucleotides and cofactors such as heme and iron–sulfur clusters (Gao and Wang et al., 2022; Maureen, 2024).

Mitochondria are particularly abundant in tissues with high energy demands, including brain and muscle. In neurons, they are actively transported to axon terminals, where synaptic transmission requires substantial ATP supply (Devine and Kittler, 2018). In muscle cells, mitochondria are densely localized near contractile elements, and their number increases with physical activity to meet elevated energy demand (Lin, 2016; Memme and Erlich et al., 2021). The importance of mitochondria in human health is reflected by the association of more than 40% of mitochondrial proteins with disease (Morgenstern et al., 2021). Mitochondrial

dysfunction has been implicated in several disorders, including metabolic syndrome, diabetes, cancer, cardiovascular disease, and neurodegenerative conditions (Koopman et al., 2012; Schon and DiMauro et al., 2012; Vafai and Mootha, 2012; Wallace, 2012; Oliveira et al., 2021; Zong et al., 2024).

3.4 Mitochondrial biogenesis

Mitochondrial biogenesis refers to the process by which cells increase their mitochondrial number (Sanchis et al., 2014; Valero, 2014). It is a complex process that requires coordinated interaction between the mitochondrial and nuclear genomes (LeBleu et al., 2014). It was first discovered by John Holloszy in the 1960s (Holloszy, 2011).

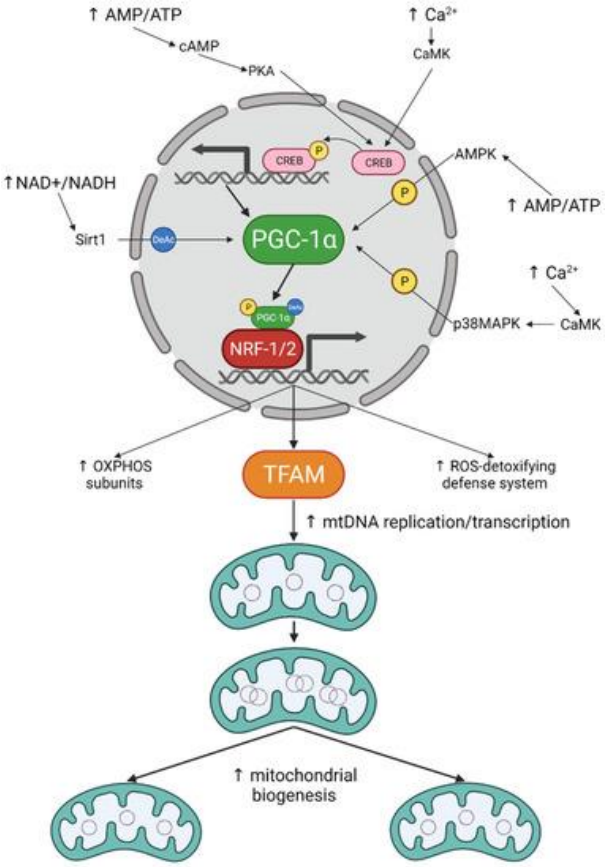


Figure 4: Mitochondrial biogenesis signaling cascades (Adapted from Cardanho-Ramos et al., 2021)

This process is triggered by various signals during periods of cellular stress, such as ATP depletion, or in response to environmental stimuli like aerobic exercise (Boushel et al., 2014; Sanchis et al., 2014; Valero, 2014). Mitochondria are generated through the transcription and translation of genes found in both the nuclear and mitochondrial genomes. Most mitochondrial proteins are encoded by the nuclear genome, whereas the mitochondrial genome provides components of the electron transport chain, as well as mitochondrial rRNA and tRNA (Mishra and Chan, 2016).

3.4.1 Fusion and fission

Mitochondria are highly adaptable organelles that can alter their shape through fission and fusion events. These opposing processes enable the mitochondrial network to undergo continuous remodeling and maintain cellular function (Bertholet et al., 2016; Mishra and Chan, 2016). Maintaining a balance between mitochondrial fission and fusion is essential for proper organization of the mitochondrial network during biogenesis, and this balance is thought to play a significant role in muscle adaptation to physiological stress (Youle et al., 2012). Mitochondrial fusion and fission are both controlled by GTPases of the dynamin family (Youle et al., 2012; Mishra and Chan, 2016).

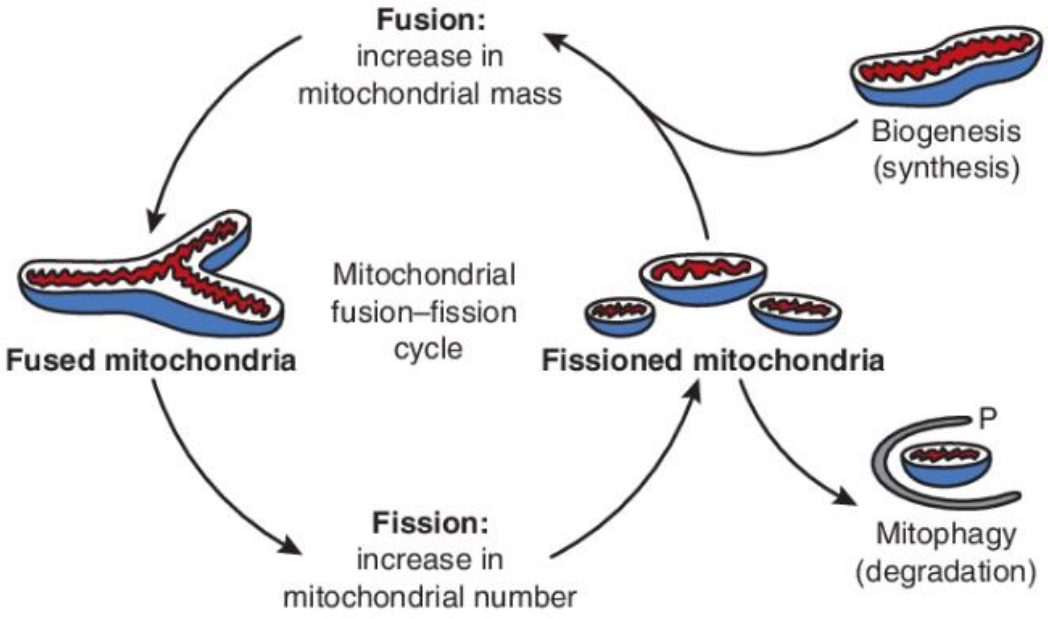


Figure 5: The processes of fusion and fission (adopted from Seo et al., 2010)

Mitochondrial fission is regulated by the protein Drp1, which assembles into a spiral structure around the mitochondrion and constricts it, leading to the division of both the outer and inner mitochondrial membranes (Bo et al., 2010). Mitochondrial fusion is regulated by various membrane-anchored dynamin-related proteins, which act at different mitochondrial membranes to facilitate the merging of the organelle's compartments (Youle et al., 2012). Fusion of the outer mitochondrial membrane is mediated by the proteins Mfn1 and Mfn2, while fusion of the inner mitochondrial membrane is facilitated by Opa1 (Youle et al., 2012; Mishra and Chan, 2016).

3.4.2 Regulation

The key regulators of mitochondrial biogenesis include PGC-1α AMPK, ERR, NRF,SIRT-1, TFAM, and TIM23. The activation of mitochondrial DNA is primarily controlled by the PGC-1 family of proteins, with PGC-1α serving as the master regulator of mitochondrial biogenesis (Johri et al., 2013; Sanchis et al., 2014; Valero, 2014). The biogenesis pathway is initiated by the activation of PGC-1α via phosphorylation and deacetylation. PGC-1α then co-activates nuclear respiratory factor 1 (NRF1). The nuclear respiratory factors (NRFs) subsequently activate mitochondrial transcription factor A (TFAM), which is directly responsible for transcribing nuclear-encoded mitochondrial proteins(Figure 6) (Johri et al., 2013; Cameron et al., 2016; Cameron et al., 2017; dos Santos et al., 2018). The translation of mitochondrial DNA (mtDNA)-encoded genes into proteins is supported by specific translation factors produced from nuclear DNA. Mitochondrial protein levels are further regulated by the translational activator of cytochrome c oxidase 1, which binds to mitochondrial mRNA and enhances translation (Yokokawa et al., 2018).

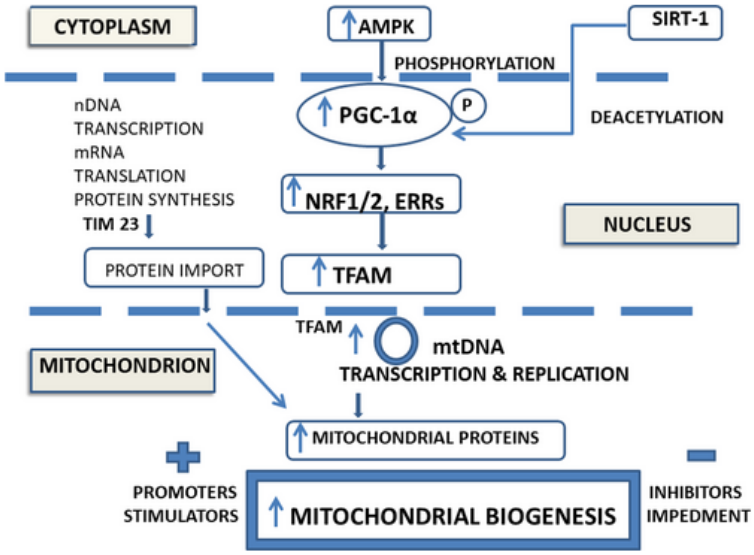


Figure 6: Mitochondrial biogenesis: The central role of PGC-1α activationand the contribution of proteins encodedby both nuclear and mitochondrialgenomes (nDNA and mtDNA) to enhancethe mitochondrial proteins content.AMPK, 5' adenosine monophosphate-activated protein kinase; ERR, theoestrogen-related receptor; NRF, thenuclear respiratory factor; PGC-1α,the peroxisome proliferator-activatedreceptor-γ coactivator-1α; SIRT-1, thesilent information regulator-1; TFAM, thetranscription factor α; TIM 23, translocase (Adapted from Popov, 2020)

AMP-activated protein kinase (AMPK) regulates mitochondrial biogenesis by sensing cellular energy depletion and activating peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), particularly in energy-demanding tissues such as muscle (Jornayvaz et al., 2010;

Scarpulla, 2011; Johri et al., 2013). Upon activation, AMPK promotes PGC-1α activity and enhances downstream nuclear respiratory factor (NRF)-mediated transcription, thesupporting mitochondrial biogenesis (Jornayvaz et al., 2010; Scarpulla, 2011; Johri et al., 2013).

Most mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytosol as precursor proteins, and imported into mitochondria through specific translocase systems. Preproteins containing amino-terminal targeting sequences are recognized by the TIM23 translocase and directed into the mitochondrial matrix or sorted to other mitochondrial compartments, including the inner mitochondrial membrane (Demishtein-Zohary et al., 2017; Moulin et al., 2019). The outer mitochondrial membrane forms the interface between mitochondria and the cytosol and contributes to mitochondrial dynamics and organelle interactions (Bruggisser et al., 2017). Common markers of mitochondrial biogenesis include increased mitochondrial DNA (mtDNA) copy number, a higher mtDNA/nuclear DNA ratio, and elevated expression of mitochondrial genes (Zou et al., 2016; Andres et al., 2017; Golpich et al., 2017).

3.5 Role of PGC-1α

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a protein that in humans is encoded by the PPARGC1A gene. It acts as a transcriptional coactivator, interacting with multiple transcription factors to modify gene expression without directly binding specific DNA sequences. It is involved in processes such as adaptive thermogenesis, mitochondrial biogenesis, glucose and fatty acid metabolism, fiber type switching in skeletal muscle, and heart development (Abu et al., 2023; Villena, 2015; Tan et al., 2016). It is a member of the PGC family of transcriptional regulators. In humans, the gene is located on chromosome 4 and encodes a protein composed of 798 amino acids, with a molecular weight of 91 kDa. The protein can be divided into several functional regions, including an activation domain, an inactivation domain, short serine/arginine-rich stretches, and an RNA recognition motif. PGC-1α also contains two putative nuclear localization signals and is found within the cell nucleus (Abu et al., 2023). It is abundantly expressed in tissues with high mitochondrial content and active oxidative metabolism, including brown adipose tissue, heart, and skeletal muscle. It is also present at elevated levels in the brain and kidney, but its expression is low in the liver and very low in white adipose tissue (Villena, 2015; Tan et al., 2016).Notably, PGC-1α itself does not have histone acetyltransferase (HAT) activity (Bost et al., 2015).

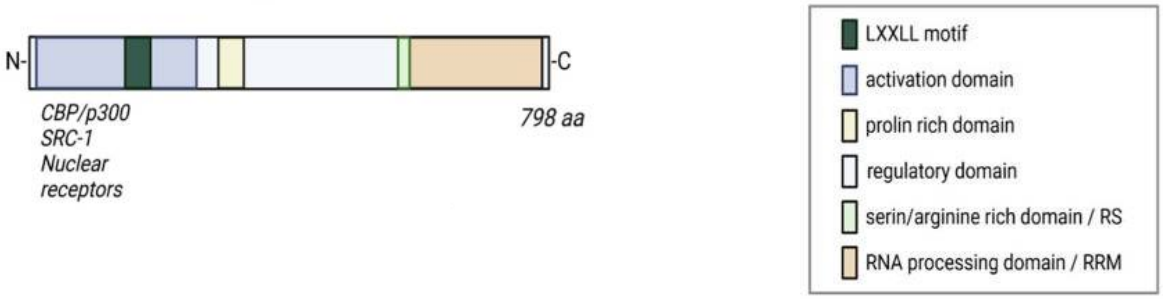


Figure 7: Structure of PGC-1α (Adapted from Jumper et al., 2021)

PGC-1α contains three LXXLL leucine-rich motifs near its N-terminal activation and regulatory regions, which allow it to interact with several nuclear receptors and transcription factors, including PPARα, estrogen receptor (ER), NRF-1, and NRF-2 (Kong et al., 2022). Its C-terminal region contains serine/arginine-rich and RNA-processing domains, suggesting a role in mRNA processing and gene expression regulation (Figure 7) (Chinsomboon et al., 2009; Yoshioka et al., 2009; Norrbom et al., 2011; Ruas et al., 2012; Mouler et al., 2013; Martinez et al., 2016; Chambers et al., 2020).

PGC-1α is considered a master regulator of mitochondrial biogenesis and oxidative metabolism. It coactivates several transcription factors, including ERR, PPARγ, NRF-1, and NRF-2, thereby promoting mitochondrial gene expression and respiratory function (Halling et al., 2019; Fontecha et al., 2020). PGC-1α is activated through AMPK-mediated phosphorylation or SIRT1-mediated deacetylation, after which it stimulates NRF-1 and NRF-2 to increase the expression of mitochondrial transcription factor A (TFAM), a key regulator of mitochondrial DNA transcription and replication (Scarpulla et al., 2008; Olesen et al., 2010; Vicente, 2016). Through the PGC-1α–NRF-1/2 pathway, the expression of genes encoding mitochondrial complex I and cytochrome c is enhanced, thereby supporting oxidative phosphorylation (OXPHOS) (Taherzadeh Fard et al., 2011). In addition to mitochondrial biogenesis, PGC-1α also regulates mitochondrial dynamics, mitophagy, carbohydrate metabolism, and lipid metabolism, linking it to metabolic disorders such as obesity, diabetes, and cardiomyopathy (Vandenbeek et al., 2018).

3.6 Nicotine

Tobacco is recognized as the most common and leading cause of preventable cancers (WHO Data. Tobocco Fact Sheet; No.339). Nicotine is a naturally occurring alkaloid that was first identified in *Nicotiana tabacum* by Posselt and Reimann in 1828, and later synthesized for the first time by Pictet and Rotschy in 1904 (Carmody et al., 2018; Sansone et al., 2023; Tushingham et al., 2018). Nicotine is produced in the roots of the plant and accumulates in its leaves. It is also present in smaller quantities in other members of the Solanaceae family, including potatoes, tomatoes, eggplants, and chili peppers (Samuels et al., 2015; Jassbi et al., 2017). Nicotine is a small molecule consisting of a pyridine ring and a pyrrolidine ring (Figure 8), and it exists in two enantiomeric forms: (i) levorotatory ([-]-nicotine), which is the natural form, and (ii) dextrorotatory ([+]-nicotine). In tobacco, nicotine accounts for 0.6% to 3% of the plant's dry weight and is the main addictive substance in tobacco smoke and other nicotine-containing products (Berridge et al., 2010, Wittenberg et al., 2020; Le foll et al., 2022; Tiwari et al., 2020, Prochaska et al., 2016). In its pure state, it is a colorless, clear liquid with a distinctive odor, which tends to turn brown when exposed to air. Nicotine is water-soluble and separates more readily from organic solvents (Mishra et al., 2015). Nicotine is a combustible substance that can cause developmental toxicity. It is capable of causing toxicity through skin absorption as well as inhalation. During combustion, nicotine can also generate toxic oxides of nitrogen. Its molecular weight is 162.23 g/mol. Nicotine exposure impacts numerous organ systems and the majority of nicotine effects are mediated by the activation of nAChRs in a wide variety of neuronal and non-neuronal tissues (Sansone et al., 2023). Nicotine is a dibasic compound, and its availability and absorption in the human body depend on the pH of the solution (Mishra, et al., 2015).

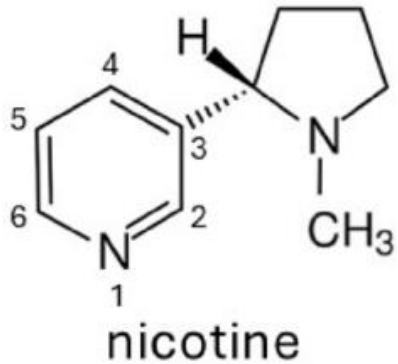


Figure 8: Structure of nicotine (Adapted from Pankow et al., 2025)

The main sites of nicotine absorption are the oral mucosa, lungs, skin, and gastrointestinal tract. When the pH of a solution rises, the concentration of uncharged, lipophilic nicotine increases.

In this state, nicotine is able to readily cross all biological membranes. After nicotine is ingested, it is absorbed and then metabolized in the liver (Mayer, 2014; Alkam et al., 2019; Luppacchini et al., 2020).

3.7 Nicotine and Mitochondria

The effect of nicotine on mitochondrial oxygen consumption indicates a direct impact on respiratory chain complexes. Nicotine binds to complex I and inhibits its NADH-ubiquinone reductase activity. Notably, nicotine has a higher affinity for mitochondria than for membrane-bound nAChRs. Nicotine binding results in inhibition of electrons flowing from NADH to complex I which is linked to a detectable decrease in oxygen consumption by mitochondria. The competition between nicotine and NADH leads not only to decreased complex I activity, but also to reduced superoxide generation (Malinska et al., 2019).

Decreased OXPHOS functionality seemed to be caused rather by nicotine modulating enzymatic activity of respiratory complexes than by its effect on the level of OXPHOS complexes. Even a high dose of nicotine had no effect on the levels of individual OXPHOS subunits (Guo et al., 2022).

Nicotine has also demonstrated direct effects on the mitochondrial respiratory chain in an anoxia/reoxygenation model. When nicotine was applied before anoxia and reoxygenation, it produced a concentration-dependent protective effect in isolated brain mitochondria. Notably, if nicotine was administered after anoxia but just prior to reoxygenation, it did not preserve mitochondrial function (Malinska, et al., 2019).

The effect of nicotine on the mitochondrial respiratory chain has been observed in certain neurodegenerative diseases such as Parkinson’s Disease. The beneficial effect symptoms of neurodegenerative of nicotine in smokers are: Nicotine is considered as an antioxidant, it could have a direct effect on OXPHOS complexes and in this way modulate mitochondrial bioenergetics (Malinska et al., 2019).

Intracellular Reactive Oxygen Species formation is a natural and metabolic process, occurring in different intracellular compartments including mitochondria (Holmstrom and Finkel, 2014). Nicotine was reported to modulate intracellular ROS production. Since nicotine can bind and inhibit respiratory chain complex 1, it can potentially affect respiratory chain mediated ROS production (Munro and Treberg, 2017). The nicotine treatment increases cytosolic ROS levels and leads to oxidative stress (Pelissier-Rota et al., 2015; Ande et al., 2016; Arany et al., 2016; Hall et al., 2017; Lam et al., 2016; Bodas et al., 2016).

Nicotine exerts its biological function mainly through stimulation of nAChR located in the cell membrane, which leads to an increase in intracellular Ca^{2+} levels. This change in cytosolic calcium levels may influence Ca^{2+} flux into mitochondria, which subsequently leads to changes in mitochondrial metabolism (Guo et al., 2012). Mitochondrial calcium buffering is important for decreasing cytosolic levels of these ions and is involved in the regulation of many Ca2+ dependent processes (Gergalova et al., 2012).

Nicotine can directly affect mitochondrial respiration by acting on the electron transport chain. It has been reported to bind mitochondrial complex I and inhibit its NADH–ubiquinone reductase activity, thereby reducing electron transfer from NADH to complex I and decreasing mitochondrial oxygen consumption. Interestingly, nicotine shows a higher affinity for mitochondria than for membrane-bound nicotinic acetylcholine receptors (nAChRs). Competition between nicotine and NADH at complex I may reduce complex I activity and also decrease superoxide generation under certain conditions (Malinska et al., 2019).

The reduction in oxidative phosphorylation (OXPHOS) appears to be mainly due to nicotine-mediated changes in the enzymatic activity of respiratory complexes rather than changes in the expression of OXPHOS subunits. Even high doses of nicotine have been reported not to alter the levels of individual OXPHOS complex proteins (Guo et al., 2022).

Nicotine has also shown direct effects on the mitochondrial respiratory chain under anoxia/reoxygenation conditions. In isolated brain mitochondria, nicotine treatment before anoxia and reoxygenation produced concentration-dependent protection. However, when nicotine was administered after anoxia but immediately before reoxygenation, it failed to preserve mitochondrial function, suggesting that the timing of nicotine exposure is important for its mitochondrial effects (Malinska et al., 2019).

The influence of nicotine on mitochondrial respiration has also been discussed in relation to neurodegenerative diseases such as Parkinson’s disease. Some reports suggest that nicotine may exert antioxidant-like effects and directly modulate OXPHOS complexes, thereby influencing mitochondrial bioenergetics (Malinska et al., 2019).

ROS are naturally produced during cellular metabolism, particularly in mitochondria (Holmstrom and Finkel, 2014). Since nicotine can bind and inhibit complex I, it may influence mitochondrial ROS generation (Munro and Treberg, 2017). However, nicotine has also been

reported to increase cytosolic ROS levels and promote oxidative stress in different experimental systems (Pelissier-Rota et al., 2015; Ande et al., 2016; Arany et al., 2016; Bodas et al., 2016; Lam et al., 2016; Hall et al., 2017).

Nicotine mainly exerts its cellular effects through activation of nAChRs located on the plasma membrane, leading to increased intracellular Ca^{2+} levels. Altered cytosolic calcium can influence calcium uptake into mitochondria and thereby affect mitochondrial metabolism (Guo et al., 2012). Mitochondrial calcium buffering is important for maintaining cytosolic calcium homeostasis and regulating several calcium-dependent cellular processes (Gergalova et al., 2012).

4. MATERIALS AND METHODS

4.1 Chemicals

Nicotine (Cat no: N3876) was obtained from Sigma Aldrich, USA. MitoTracker™ Green FM (Cat no: M7514) was obtained from Invitrogen, USA. All the other chemicals and biochemicals used were of quality grade.

4.2 Other consumables used

Table 4.1: List of consumables used in the study

Name of Consumables	Catalogue Number	Company Name
Dulbecco's modified eagle medium	12800017	Gibco, NY, USA
10% fetal bovine serum	10270-106	Gibco, NY, USA
1% penicillin- streptomycin	A001	Himedia, Maharashtra, India
2 mM alaine-glutamine	G8541	Sigma Aldrich, MO, USA
60 mm dishes	TCP007	Himedia, India
RIPA lysis buffer	20-188	EMD Millipore Corp, MA, USA
Protease phosphatase inhibitor	PPC1010	Sigma Aldrich, MO, USA
Qubit™ Protein and Protein BR Assay kit	Q33211	Invitrogen, Thermo Fischer Scientific, California, US
Qubit 4 Flurometer	A5068	Invitrogen, Thermo Fischer Scientific, California, US
30% Acrylamide Bis-acrylamide	ML037-100ML	HIMEDIA, Maharashtra, India
10%Ammonium Persulphate	A378-25G,	Sigma Aldrich, MO, USA
Non- Reducing Lane Marker Sample Buffer	39001	Thermo Fischer Scientific, California, US
Tween 20	P9416	Sigma Aldrich, MO, USA
SuperSignal™ West Femto Maximum Sensitivity Substrate	34094	Thermo Fischer Scientific, California, US
Restore™ Western Blot Stripping Buffer	21059	Thermo Fischer Scientific, California, US

4.3 Antibodies

Table 4.2: List of primary and secondary antibodies used for western blotting

Name of antibody	Catalogue number	Company name	Dilution	Temperature for incubation
β-actin	#AC026	ABClonal (BioAb), New Taipel City, Taiwan	1:20,000	4°C
PGC-1α	MA5-32563	Invitrogen, Cakifornia,USA	1:1000	4°C
Anti-rabbit IgG, HRP-linked Antibody	#7074	Cell Signaling Technologies, Danvers, Massachusetts, USA	1:2000	Room temperature

4.4 Cell line

The SH-SY5Y cell line is a human-derived, thrice-cloned subline originating from the SK-N-SH neuroblastoma cell line. This cell line was purchased from NCCS, Pune. Cells were cultured in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 2 mM alanine-glutamine. Cultures were maintained at 37°C in an Eppendorf CellXpert C170 CO_2 incubator with 95% humidity and 5% CO_2.

4.5 Preparation of DMEM Cell Culture Media

The DMEM cell culture media was prepared by dissolving powdered DMEM in 1L of sterilized water, followed by supplementation with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM alanine-glutamine. The resulting mixture was filtered through a 0.22 μm membrane filter and stored at 4°C until use.

4.6 MitoTracker Green FM assay

MitoTracker™ Green FM are widely used for staining mitochondria of live cells depending, on the organelle lipid content (mitochondrial mass) and oxidative activity (mitochondrial membrane potential). Cells were seeded onto 60 mm dishes. After attaining 85-90% confluency. The existing culture medium from the cells were gently aspirated. Cells were then

changed to serum free medium. The cells were then challenged with nicotine (0.1μM) and MitoTracker™ Green FM dye (200 nM) was added, then incubated for 4 hours at 37^{o} C and 5% CO_2. After the incubation period, the cells were washed thrice with serum free media and then imaging was carried out using an Evos FLoid cell imaging station fluorescent microscope, Life technologies, USA. Images were analysed using Image J 1.53t (NIH) software and statistical analysis was done by using GraphPad Prism 5.

Table 4.3: Experimental designs

Group name	**Concentration**
Group 1	Basal
Group 2	Cells challenged with nicotine (0.1μM) for 4 hours

4.7 Experimental Design

SH-SY5Y cells were cultured in 60 mm dishes. Upon reaching 80% confluency, the medium was removed and replaced with DMEM containing 0.5% FBS for low serum conditions, followed by treatment with 0.1 μM nicotine. After a 4-hour incubation, the cells were placed on ice, and the medium was discarded. Cells were washed with 1 ml of ice-cold PBS (pH 7.4), and after PBS removal, 80 μl of RIPA lysis buffer supplemented with a protease phosphatase inhibitor cocktail was added to each dish. The dishes were kept on ice for 10 minutes, and cells were scraped using a cell scraper. The lysate was then transferred to a fresh 1.5 ml centrifuge tube and centrifuged at 4°C at 15000 rpm for 15 minutes (Eppendorf Centrifuge 5425 R). The supernatant was collected and transferred to a new tube. Protein concentration was determined using the Qubit assay.

4.8 Protein Estimation by Qubit BR (Broad Range) Assay

Protein concentration was quantified using Qubit™ Protein and Protein Broad Range (BR) Assay kit as per the manufactures's instruction using Qubit 4 Fluorometer.

1. Standards were prepared by adding 10 μl of each Qubit standard (Standard 1 and Standard 2) and 160 μl of Qubit Protein BR Assay Buffer to separate designated tubes. The solutions were mixed thoroughly by pipetting up and down.

2. For sample preparation, 10 µl of each protein lysate was transferred into the assigned sample tubes. To each tube, 160 µl of Qubit protein BR assay buffer was added, bringing the final volume to 170 µl. The samples were thoroughly mixed.
3. Sample buffer blank was prepared by first adding 10 µl of RIPA buffer to a tube, then adding 160 µl of Qubit protein BR assay buffer to bring the total volume to 170 µl. The mixture was then mixed by pipetting.
4. Lastly, 30 µl of Qubit protein BR assay reagent was added to each sample tube, standard tube, and the sample buffer blank.
5. All solutions were promptly mixed by pipetting and vortexing for 5–7 seconds, resulting in a final volume of 200 µl in each tube.
6. After incubating at room temperature for 10 minutes, protein concentration was measured using the Qubit 4 Fluorometer.

4.9 SDS PAGE Electrophoresis

4.9.1 Reagents

- 30% Acrylamide Bis-acrylamide
- 10% SDS: 5g of SDS was mixed with 45 ml of distilled water and made up to a volume of 50 ml.
- 0.5 M Tris HCL (pH-6.8): 19.7 g of Tris HCL dissolved in 150 ml of distilled pH was adjusted to 6.8 by using 15N NaOH and made up to 250 ml.
- 1.5 M Tris HCL (pH-8.8): 118.2 g of Tris HCL was dissolved in 250 ml of distilled water. The pH was adjusted 8.8 by using 15N NaOH and made up to 500 ml.
- 10% Ammonium Persulphate (freshly prepared): 0.1g of APS was dissolved in 1 ml of distilled water.
- Running buffer: 30.30 g of Tris base: 144.10 g of glycine, 10g of SDS were dissolved in 1L of distilled water.
- TEMED (N,N,N’,N’-Tetramethylethylenediamine)

4.9.2 Preparation of 8% Polyacrylamide gel

The gel was prepared by combining distilled water, 30% Acrylamide Bis-acrylamide, Tris-HCl buffer (pH 8.8), and 10% SDS. To this mixture, 10% APS and TEMED were added and mixed thoroughly. The solution was then poured between the glass plates of a vertical gel assembly and allowed to polymerize.

4.9.3 Preparation of 4% Polyacrylamide gel

The gel was prepared by mixing distilled water, 30% Acrylamide Bis-acrylamide,Tris-HCl buffer (pH 6.8), and 10% SDS. After adding 10% APS and TEMED, the solution was mixed thoroughly and poured between the glass plates of the vertical gel assembly. The gel was then allowed to polymerize.

4.9.4 Sample Preparation

The protein samples were mixed with Non – Reducing Lane Marker Sample Buffer. Following the mixing of the dye and the sample, heat the sample for 10 minutes on 90°C. Rest the samples for 10 minutes on ice, following heating. Then load the mixture onto the gel.

4.9.5 Electrophoresis Procedure

Electrophoresis was performed using a BIORAD PowerPac™ HC. The run was initiated at 80 V for 20 minutes, then the voltage was increased to 120 V and maintained until the dye front reached the bottom of the gel.

4.10 Immunoblotting

4.10.1 Reagents

- 10X Tris Buffered Saline (TBS) was formulated by dissolving 24 g of Tris HCL and 88g of NaCl in 800 ml of deionized water. The pH is adjusted to 7.6 with 15N NaOH and the total volume is made up to 1L. This was diluted to 1X before use as required.
- 25X Tris Glycine Transfer Buffer (pH-8.3) was made by dissolving 9.1 g of Tris base and 45 g of glycine in distilled water and made up to 500 ml. This was diluted to 1X before use as required.
- 1X Tris Buffered Saline with Tween 20 (TBST) was formulated by dissolving 1ml of Tween 20 and 100 ml of 10X TBS and the final volume is made up to 1L with distilled water.

4.10.2 Procedure

Following electrophoresis, proteins were transferred to a nitrocellulose membrane (0.45μm) at 100 V for 2 hours at 4°C using a Bio-Rad transfer apparatus. The membrane was then blocked

with 5% BSA in TBS for 1 hour and 30 minutes at room temperature. After blocking, the membrane was incubated overnight at 4°C with primary antibodies against β-actin and PGC1-α. Washing was performed three times with TBST (Tris Buffered Saline containing 0.1% Tween 20), each wash lasting 10 minutes. The membrane was then incubated with a secondary antibody, diluted according to the manufacturer's instructions, for 1.5 hours at room temperature. After incubation, the membrane was washed again with TBST thrice for 10 minutes each. Chemiluminescent detection was carried out using a Bio-Rad XRS+ Chemidoc system and SuperSignal™ West Femto Maximum Sensitivity Substrate. Blot were analysed using ImageJ 1.53t (NIH) software.

4.10.3 Stripping

Stripping was performed using Restore™ Western Blot Stripping Buffer. The buffer was pre-warmed to 50°C and then applied to the membrane for 20 minutes at room temperature. This was followed by a 10-minute incubation with agitation. The buffer was removed, and the membrane was washed thrice with TBST, each wash lasting 10 minutes. The membrane was then re-blocked with 5% BSA in TBS for 1 hour at room temperature.

4.10.4 Image J Analysis

Band intensities of blots were analysed using ImageJ (NIH). Images were converted to grayscale, and individual bands were selected for densitometric analysis. Background signals were subtracted, and intensity values were normalized to the loading control (β-actin). These normalized values were then used to assess changes in protein expression following nicotine treatment.

4.10.5 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 5. Group comparisons were carried out with Paired t-test, and statistical significance was represented in the graphs as $p < 0.05$.

5. RESULTS

5.1 MitoTracker Green FM Analysis on Basal and Nicotine Challenged SH-SY5Y Cells

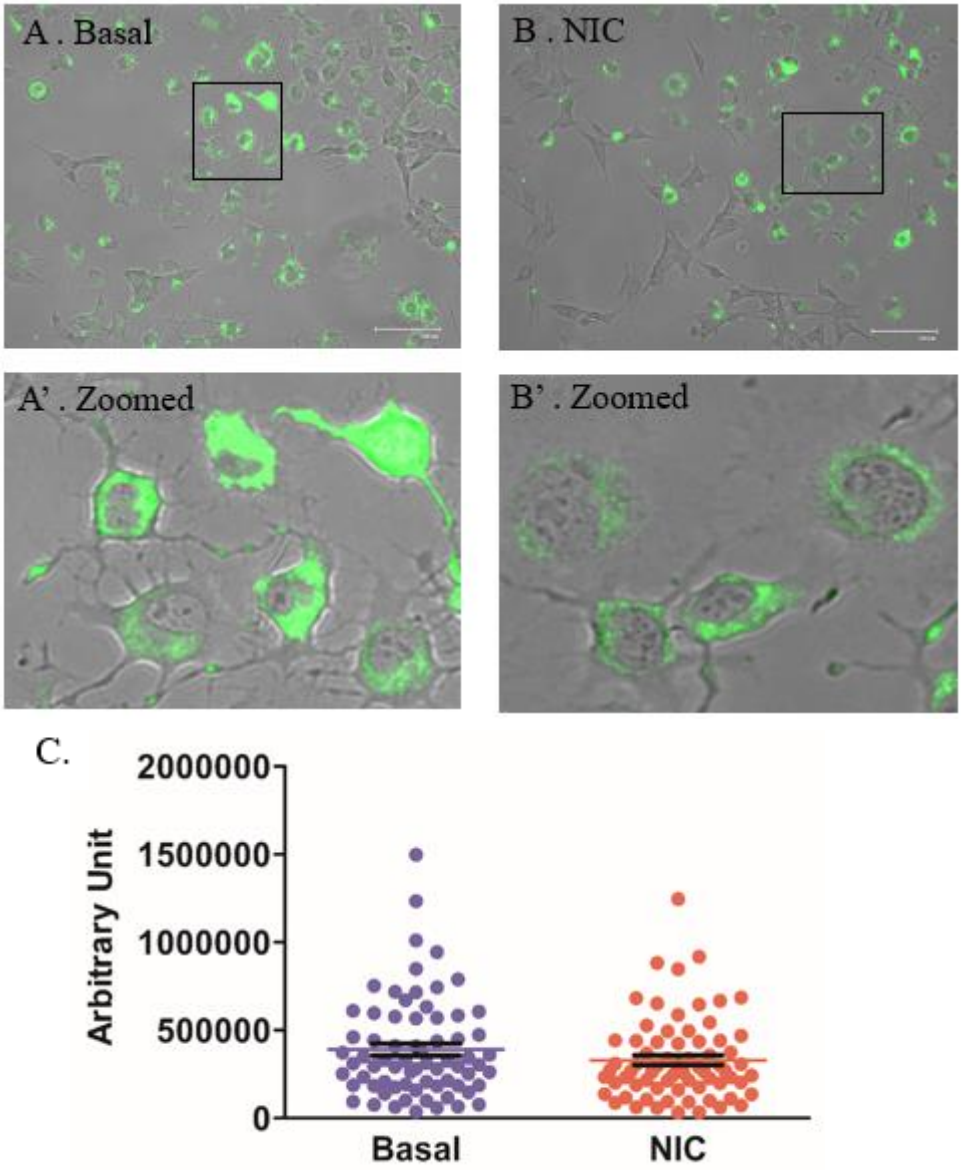


Figure 9: (A-B) Representative fluorescence images of basal and nicotine challenged SH-SY5Y cells labelled with MitoTracker GreenFM to assess mitochondrial content. (A'- B') Zoomed images highlighting mitochondrial labelling. (C) MitoTracker Green fluorescence intensity expressed as arbitrary units for individual cells (70 cells) in basal and nicotine- challenged groups. Data are presented as scatter plots. Statistical analysis was done using GraphPad Prism 5.

Mitochondrial intensity was analysed using MitoTracker Green ^{FM} dye. Basal cells showed normal mitochondrial fluorescence intensity at 4th hour (Figure 9.A). Addition of nicotine (0.1μM) for 4 hours shows a decrease in mitochondrial fluorescence intensity at 4th hour (Figure 9.B)

5.2 Changes in PGC-1α Expression in Basal and Nicotine Challenged SH-SY5Y Cells by Western Blottig

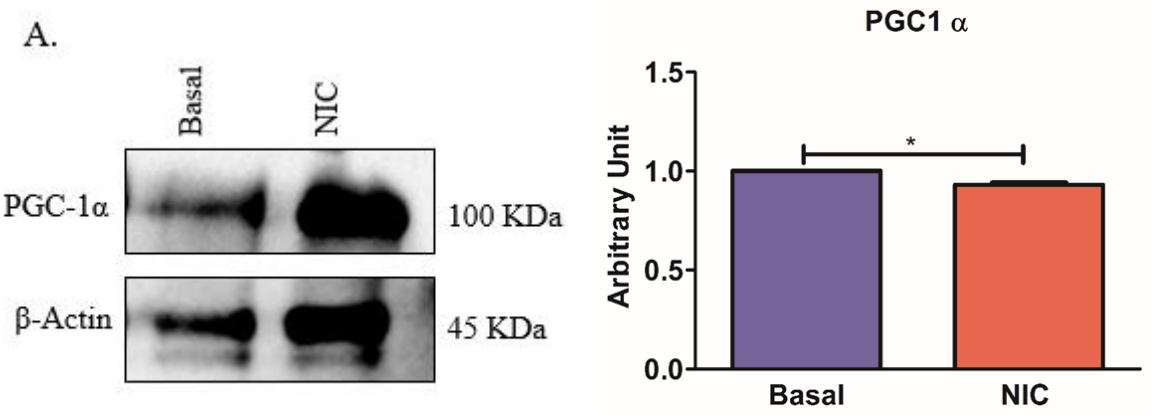


Figure 10: (A) Representative western blots images showing changes in protein expression in SH-SY5Y cells.β-Actin was used as the loading control. (B) The protein densities were analyzed using Image J (NIH)1.53t software. * Statistical significance between Basal and NIC (p value < 0.05) for PGC-1α was done using GraphPad Prism 5.

PGC-1α expression in nicotine challenged cells were analysed using western blotting (Figure 10.A). PGC-1α protein showed basal expression at 4th hour. Addition of nicotine (0.1μM) for 4 hours caused decrease (p=0.026) in protein expression of PGC-1α at 4th hour.

6. DISCUSSION

The present study examined the early effect of nicotine exposure on mitochondrial status in SHSY5Y neuroblasts using MitoTracker Green FM staining and PGC-1α protein expression analysis. Cells maintained under basal conditions showed normal mitochondrial fluorescence intensity at the 4th hour, indicating preserved mitochondrial staining and distribution under control conditions. Exposure to nicotine at 0.1 µM for 4 h produced a mild reduction in MitoTracker Green FM fluorescence intensity. Although the reduction was modest, it suggests that acute nicotine exposure may alter mitochondrial content, mitochondrial staining intensity, or mitochondrial organization in neuronal cells.

MitoTracker Green FM is commonly used to assess mitochondrial mass or mitochondrial abundance, as it stains mitochondria relatively independent of mitochondrial membrane potential (Gautham et al., 2018; Bergman et al., 2025). Therefore, the observed reduction in fluorescence intensity following nicotine exposure may reflect an early decrease in mitochondrial content or altered mitochondrial integrity. However, since fluorescence-based mitochondrial staining can also be influenced by dye uptake, mitochondrial morphology, and cell density, this result should be interpreted as an indication of altered mitochondrial status rather than definitive evidence of mitochondrial loss.

The Western blot analysis showed that nicotine exposure significantly decreased PGC-1α protein expression at the 4th hour compared with basal control cells. PGC-1α is a major transcriptional coactivator involved in mitochondrial biogenesis, oxidative metabolism, and cellular energy regulation (Abu et al., 2023; Villena, 2015; Tan et al., 2016). It activates downstream transcription factors such as NRF-1 and NRF-2, which in turn regulate mitochondrial transcription factor A (TFAM), a key factor required for mitochondrial DNA replication and transcription (Olesen et al., 2010; Johri et al., 2013; Cameron et al., 2016; Vicente, 2016; Cameron et al., 2017; dos Santos et al., 2018). Therefore, the reduction in PGC-1α expression suggests that nicotine may suppress mitochondrial biogenic signalling in neuronal cells even during short-term exposure.

The decrease in PGC-1α also supports the MitoTracker Green FM finding, as reduced mitochondrial biogenesis could contribute to lower mitochondrial fluorescence intensity. Since PGC-1α is activated by energy-sensing pathways such as AMPK and SIRT1, its reduced expression may indicate impaired mitochondria (Zhang and Liang et al., 2019). In neuronal

cells, where mitochondrial function is essential for ATP production, calcium buffering, and synaptic activity, suppression of PGC-1α may compromise the ability of cells to maintain mitochondrial homeostasis during stress.

Previous studies have shown that nicotine can directly affect mitochondrial respiration by interacting with respiratory chain complex I and modulating NADH-linked electron transport, oxygen consumption, and reactive oxygen species generation (Malinska et al., 2019). Nicotine has also been reported to alter oxidative phosphorylation mainly through changes in respiratory complex activity rather than by reducing the expression of OXPHOS subunits (Guo et al., 2022). In addition, nicotine can activate nicotinic acetylcholine receptors and increase intracellular Ca^{2+} levels, which may influence mitochondrial calcium handling and metabolism (Gergalova et al., 2012; Guo et al., 2012). Thus, the reduction in PGC-1α observed in the present study may be part of an early mitochondrial response to nicotine-induced metabolic or calcium-associated stress.

The present findings indicate that even a short exposure to low-dose nicotine can influence mitochondrial regulatory mechanisms in neuronal cells. The mild decrease in MitoTracker Green FM fluorescence, together with the significant reduction in PGC-1α expression, suggests that nicotine may impair mitochondrial biogenesis or reduce mitochondrial adaptive capacity at an early time point. This is relevant because mitochondrial dysfunction is increasingly associated with neuronal vulnerability, oxidative stress, and neurodegenerative changes.

However, the study has certain limitations. The mitochondrial fluorescence analysis provides supportive but not conclusive evidence of mitochondrial alteration. Further studies using additional mitochondrial markers such as TOM20, TFAM, NRF-1, COX IV, mitochondrial membrane potential assays, ATP estimation, ROS measurement, and oxygen consumption analysis are underway in the laboratory and would provide a more complete understanding of nicotine-induced mitochondrial dysfunction.

In conclusion, the present study suggests that acute nicotine exposure reduces PGC-1α expression and mildly decreases mitochondrial fluorescence intensity in neuronal cells. These findings indicate that nicotine may interfere with mitochondrial biogenesis and early mitochondrial homeostasis. The data provide preliminary evidence that nicotine can negatively affect mitochondrial regulatory pathways in neuronal cells, supporting the need for further investigation using additional mitochondrial functional assays.

7. SUMMARY AND CONCLUSION

This study evaluated the early effect of acute nicotine exposure on mitochondrial homeostasis in SH-SY5Y neuronal cells using MitoTracker Green FM staining and Western blot analysis of PGC-1α. Exposure to 0.1 μM nicotine for 4 hours resulted in a modest reduction in mitochondrial fluorescence intensity compared with untreated control cells, suggesting an early alteration in mitochondrial content, integrity, or organization.

Western blot analysis showed a significant decrease in PGC-1α protein expression following nicotine treatment. Since PGC-1α is a key regulator of mitochondrial biogenesis and metabolic adaptation, its downregulation indicates impaired mitochondrial biogenic signaling. Although the change in MitoTracker fluorescence was mild, the marked reduction in PGC-1α suggests that nicotine can affect mitochondrial regulatory pathways even during short-term exposure.

Overall, these findings indicate that acute low-dose nicotine exposure may compromise mitochondrial homeostasis in neuronal cells by suppressing mitochondrial biogenesis-associated signaling. This supports the possibility that mitochondrial dysfunction contributes to nicotine-induced neuronal stress.

To conclude, the findings provide preliminary evidence that nicotine can negatively affect neuronal mitochondrial health and highlight the need for further studies on mitochondrial dysfunction in nicotine-induced neurotoxicity.

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