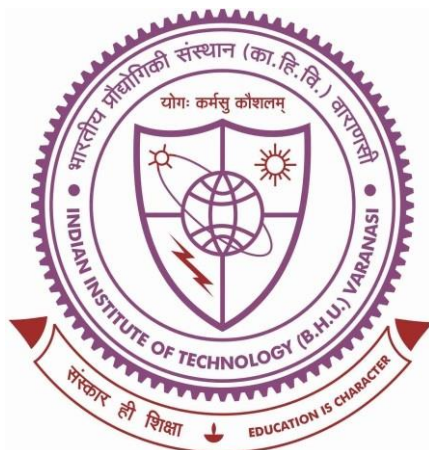


Pharmacological Intervention in dim light induced changes in circadian rhythm



Thesis submitted in partial fulfillment for the
Award of Degree

Doctor of Philosophy

By

Prabha Rajput

DEPARTMENT OF PHARMACEUTICAL ENGINEERING & TECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY
(BANARAS HINDU UNIVERSITY)
VARANASI-221005
INDIA

Roll No. 17161007

2023

CERTIFICATE

It is certified that the work contained in the thesis titled "**Pharmacological intervention in dim light induced changes in circadian rhythm.**" has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.

It is further certified that the student has fulfilled all the requirements of Comprehensive Examination, Candidacy and SOTA for the award of Ph.D. Degree.

Date:

Place: IIT (BHU), Varanasi

18/7/23
Prof. Sairam Krishnamurthy
(Supervisor)
Dept. of Engg. & Tech.
Indian Institute of Technology
(Banaras Hindu University)
Varanasi-221005 (U.P.)

DECLARATION BY THE CANDIDATE

I, Ms. Prabha Rajput, certify that the work embodied in this Ph.D. thesis is my own bonafide work and carried out by me under the supervision of Prof. Sairam Krishnamurthy from July, 2017 to July, 2023 at the Department of Pharmaceutical Engineering & Technology, Indian Institute of Technology (Banaras Hindu University), Varanasi. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

I declare that I have faithfully acknowledged and given credit to the research workers wherever their works have been cited in my work in this thesis. I further declare that, I have not willfully copied any other's work, paragraphs, text, data, results, etc. reported in the journals, books, magazines, reports, dissertations, theses, etc., or available at websites and have not included them in this Ph.D. thesis and have not cited as my own work.

Date: 18/07/2023

Place: IIT (BHU), Varanasi

Prabha

Signature of the Student

Prabha Rajput

CERTIFICATE BY THE SUPERVISOR AND HEAD OF THE DEPARTMENT

It is certified that the above statement made by the student is correct to the best of our knowledge.

18/07/23
Prof. Sairam Krishnamurthy
Dept. of Pharmaceutical Engg. & Tech.
(Supervisor)
Indian Institute of Technology
(Banaras Hindu University)
Varanasi-221005 (U.P.)

S. Hemalatha 18/7/23
Prof. Siva Hemalatha

(Head of the Department)

विभागाध्यक्ष / Head

भेषजकीय अभियांत्रिकी एवं प्रौद्योगिकी विभाग /
Department of Pharmaceutical Engineering & Technology
भारतीय प्रौद्योगिकी संस्थान / INDIAN INSTITUTE OF TECHNOLOGY
(बनारस हिन्दू विश्वविद्यालय) / (BANARAS HINDU UNIVERSITY)
वाराणसी-२२१००५ / Varanasi-221005

COPYRIGHT TRANSFER CERTIFICATE

Title of the Thesis: Pharmacological intervention in dim light induced changes in circadian rhythm.

Name of the Student: Ms. Prabha Rajput

Copyright Transfer

The undersigned hereby assigns to the Indian Institute of Technology (Banaras Hindu University), Varanasi all rights under copyright that may exist in and for the above thesis submitted for the award of the "*Doctor of Philosophy*".

Date: 18.07.2023



Signature of the Student

Place: IIT (BHU), Varanasi

Prabha Rajput

Note: However, the author may reproduce or authorize others to reproduce material extracted verbatim from the thesis or derivative of the thesis for author's personal use provided that the source and University's copyright notice are indicated.

ACKNOWLEDGEMENT

Joys on successful completion are always cherishing and everlasting. It gives the feeling of completeness on looking back over the journey and remembering all those friends and family who have helped and supported me along this long but fulfilling path. I owe my gratitude to the almighty '*Lord Shiva*' for blessings and making things all right in place. *Bharat Ratna Mahamana Pandit Madan Mohan Malaviya Ji*, Founder of BHU, for providing me such a divine platform.

Words cannot express my gratitude for my family: My mother, *Mrs. Minti Rajput*, and my Father, *Mr. Jujhar Singh Rajput*, for supporting me spiritually throughout writing this thesis and my life in general.

At this moment of accomplishment, firstly of all, I pay homage to my Ph.D. supervisor *Prof. Sairam Krishnamurthy* (Professor of Pharmacology), for giving me an opportunity to work in the field of neuropharmacology, the research area I love to work with; I am also grateful to him for grooming me not only to conduct independent research but also for acquainting me with other areas affiliated with scientific pursuits. His wide knowledge and logical way of thinking have been a great value to me. This work would not have been possible without his guidance, support and encouragement. Under his guidance, I successfully overcame many difficulties and learned a lot. His unflinching courage and conviction will always inspire me, and I hope to continue to work with his noble thoughts.

I thank my research progress evaluation committee members, *Dr. P.K. Nayak*, Department of Pharmaceutical Engineering and Technology, and *Dr. Abha Mishra*, Department of Biochemical engineering, for their valuable suggestions and comments during my Ph.D. tenure.

I thank Head of the Department (HOD) **Prof. Siva Hemalatha** and former HOD, **Prof.**

B. Mishra, Prof. S.K. Singh, Prof. Sanjay Singh and Prof. S.K. Shrivastava for their support.

It is my extreme privilege to gratuitously convey my special thanks to all the faculty members of the Department, *Prof. S. Hemlatha, Dr. A.K. Srivastava, Dr. Senthil Raja, Dr. A.N. Sahu, Dr. S.K. Mishra, Dr. Ruchi Chawla, Dr. Ashok Kumar Maurya, Dr. M.S. Muthu, Dr. G.P. Modi, Dr. P.K. Nayak, Dr. A.K. Agrawal, Dr. S.K. Jain and Dr. Vinod Tiwari, Dr. Rajnish, Dr. Deepak Kumar, Dr. Dinesh Kumar, Dr. Jairam Meena, and Dr.A.Khatri* for their kind cooperation and valuable suggestions throughout the research work.

My sincere thanks to *Mr. Ram Jiyawan, Mr. Yashwant Singh, Mr. Atul Kumar, Mr. Anand, Mr Chotelal, Mr. Virendra Kumar, Mr. Nandlal, Mr. Madanlal, Mr. Ram Hriday Pathak, Mr. Akhila Nand Upadhyay, Mr. Arun Kumar, Mr. Md. Jameel, Mr. Sunil Kumar Singh,* , and all other non-teaching staff of the department who had provided me all the necessary support while needed.

I am thankful to my Seniors/labmates/juniors, *Dr. Sukesh, Dr. Pankaj, Dr. Akanksha, Mr.Santosh Mr. Ramakrishna, Mr. Qadir, Ms. Pratigya, Ms. Shreyasi, Ms. Neha, Ms. Asha, Mr. Gajendra, Mr. Aquib, and Mr. Neeraj,* for stimulating discussions, for the sleepless nights we were working together before deadlines, and all the fun we have had in the last Six years. I would also like to express my gratitude to all those who supported me directly or indirectly for the study.

I would also like to thank *Dr. Dhananjay Kumar* for his help during my Ph.D. period. A special thanks *to Madhusudan and Tavaréz* for their assistance during my experimental work.

Finally, I am highly indebted to the almighty for showering immense blessing in gifting me my beloved parents, family, and friends.

Date: 18.07.2023

Place: IIT (BHU), Varanasi



Prabha Rajput

TABLE OF CONTENTS

Titles	Page No.
Certificate	i
Declaration by the Candidate	ii
Copyright Transfer Certificate	iii
Acknowledgement	iv-v
Table of Contents	vi-x
List of Abbreviations	xi-xii
List of Figures	xiii-xiv
List of Tables	xv
Preface	xvii
Chapter 1: Introduction	
1 Introduction	
1.1 Sleep wake cycle	
1.2 Common sleep deprivation consequences	
1.3 Difference between circadian rhythm and biological clock	
1.4 Different types of cycle	
1.4.1 Circadian rhythm	
1.4.2 Diurnal rhythm	
1.4.3 Ultradian rhythm	
1.4.4 Infradian rhythm/ Circalunar rhythm	
1.4.5 Circannual rhythm	
1.5 Circadian clock of photic and nonphotic cues	
1.6 Type of disrupted circadian rhythm disorder	
1.6.1 Delayed phase sleep wake syndrome (DSP)	
1.6.2 Early phase sleep wake syndrome (ASP)	
1.6.3 Jet lag disorder	
1.6.4 Shift work disorder	
1.6.5 Irregular sleep wake syndrome	
1.6.6 Free running (nonentrained)	
1.7 Transcription/Translation Feedback Loops (TTFL) of the Mammalian Clock	
1.8 Importance of light as a source of energy	
1.9 Circadian control of mitochondrial respiration	
1.10 Light source that we exposed in our daily life and their consequences	
1.11 Mitochondrial modulators	
1.11.1 N-Acetylcysteine (NAC)	
1.11.2 Melatonin	
1.12 Rationale	

- 1.13 Lacunae in the existing literature, relevance of the study
- 1.14 Hypothesis
- 1.15 Objectives

Chapter 2: Effect of normal and disrupted circadian rhythm on the mitochondria

2 Introduction

- 2.1 Materials and methods
 - 2.1.1 Animals and housing
 - 2.1.2 Experimental design
 - 2.1.3 Locomotor activity measurements
 - 2.1.4 Isolation of brain suprachiasmatic mitochondria
 - 2.1.5 Measurement of mitochondrial bioenergetics
 - 2.1.6 Measurement of mitochondrial DNA
 - 2.1.7 Quantification of clock genes expression
 - 2.1.8 Estimation of corticosterone
 - 2.1.9 Statistical analyses
- 2.2 Results
 - 2.2.1 Wheel running locomotors activity
 - 2.2.2 Respiratory control rate
 - 2.2.3 Mitochondrial DNA
 - 2.2.4 Clock genes
 - 2.2.5 Corticosterone
- 2.3 Discussion
- 2.4 Summary

Chapter 3: Evaluation of NAC effect on central clock related circadian rhythm

3 Introduction

- 3.1 Materials and Methods
 - 3.1.1 Animals
 - 3.1.2 Drugs
 - 3.1.3 Experimental Design
 - 3.1.4 Isolation of mitochondria from brain suprachiasmatic nuclei (SCN)
 - 3.1.5 Measurement of mitochondrial respiration
 - 3.1.6 Measurement of GSH fluorometrically
 - 3.1.7 Measurement of glutamate
 - 3.1.8 Measurement of total ATP content
 - 3.1.9 Measurement of plasma melatonin
 - 3.1.10 Estimation of corticosterone
 - 3.1.11 Quantification of clock genes
 - 3.1.12 Statistical analyses

3.2 Results

3.2.1 NAC improved the mitochondrial RCR and states in the SCN

3.2.2 NAC dose dependently improved the GSH and glutamate level in the SCN

3.2.3 NAC dose dependently improved total ATP content

3.2.4 NAC improved Melatonin

3.2.5 NAC decreases the CORT level in LL exposed mice

3.2.6 NAC dose dependently upregulated the Clock genes

3.3 Discussion

3.4 Summary

Chapter 4: Evaluation of N-acetylcysteine effect on peripheral clock related circadian rhythm

4 Introduction

4.1 Materials and methods

4.1.1 Animals

4.1.2 Drugs

4.1.3 Experimental design

4.1.4 Determination of body weight and diurnal feed intake

4.1.5 Isolation of mitochondria from the liver

4.1.6 Measurement of mitochondrial respiration

4.1.7 Staining of succinate dehydrogenase

4.1.8 Measurement of GSH fluorometrically

4.1.9 Measurement of total ATP content

4.1.10 Measurement of leptin and ghrelin

4.1.11 Estimation of plasma glucose

4.1.12 Estimation of lipid profile

4.1.13 Statistical analyses

4.2 Results

4.2.1 NAC recovered the body weight elevated by LL exposure

4.2.2 NAC restored the altered diurnal feed intake in LL exposed mice

4.2.3 NAC improved the mitochondrial RCR and states in the Liver

4.2.4 NAC improved Succinate dehydrogenase (SDH) activity in liver

4.2.5 NAC dose dependently improved the GSH in the liver

4.2.6 NAC dose dependently improved ATP level in the liver

4.2.7 NAC decreases the blood glucose level, total cholesterol, triglyceride and increases HDL in LL exposed mice

4.2.8 Leptin and ghrelin

4.3 Discussion

4.4 Summary

Chapter 5: Development of ex-vivo method for evaluation of mitochondrial modulators

5. Introduction

5.1 Materials and Methods

5.1.1 Animals

5.1.2 Chemicals

5.1.3 Experimental design

5.1.4 Determination of optimal DMSO concentration

5.1.5 Isolation of mitochondria from the liver and brain

5.1.6 Ex-vivo incubation and measurement of mitochondrial respiration

5.1.7 Mitochondrial ETC enzyme activities

5.1.7.1 NADH dehydrogenase (Complex-1) activity

5.1.7.2 Succinate dehydrogenase (Complex-2) activity

5.1.7.3 Cytochrome-C oxidase (Complex-4) activity

5.1.7.4 F1F0 ATP synthase (Complex-5) activity

5.1.7.5 Mitochondrial membrane potential (MMP)

5.1.8 Statistical analysis

5.2 Results

5.2.1 DMSO was toxic at >5% to isolated mitochondria

5.2.2 MET inhibited mitochondrial respiration; Q10 improved MET inhibited mitochondrial respiration

5.2.3 Q10 exerts a concentration-dependent effect on MET induced mitochondrial respiration

5.2.4 Q10 exerts concentration-dependent effect on MET-induced mitochondrial dysfunction on complex-1, 5 with no effect on complex-2 and 4

5.2.5 Q10 improved MET-induced changes in mitochondrial membrane potential (MMP)

5.3 Discussion

5.4 Summary

Chapter 6: Summary and conclusion

6.1 Important outcomes

6.2 Limitation of the study

6.2 Future studies

References

List of Publication from Thesis

LIST OF ABBREVIATIONS

ADP	-	Adenosine Di-Phosphate
ATP	-	Adenosine Triphosphate
BSA	-	Bovine Serum Albumin
CORT	-	Corticosterone
CT	-	Circadian Time
DMSO	-	Dimethyl sulfoxide
EGTA	-	Ethylene Glycol-bis (β -aminoethyl ether)-N, N, N', N'-Tetra acetic Acid
ETC	-	Electron transport chain
FCCP	-	Carbonyl Cyanide 4-(trifluoromethoxy) Phenylhydrazone
HEPES- 4	-	(2-HydroxyEthyl)-1-Piperazine Ethane Sulfonic acid
HPLC	-	High-performance liquid chromatography
KH ₂ PO ₄	-	Potassium Phosphate Monobasic Anhydrous
LD	-	Light Dark (12:12 h)
LL	-	Continuous dim light (24:00 h)
MET	-	Metformin
MgCl ₂	-	Magnesium Chloride
MPTP	-	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
NAD	-	Nicotinamide adenine dinucleotide
NADH	-	Nicotinamide adenine dehydrogenase
OXPHOS	-	Oxidative phosphorylation
Q10	-	Coenzyme Q10

RCR	-	Respiratory Control Rate
RSV	-	Resveratrol
RT	-	qPCR- Real-Time Polymerase Chain Reaction
SCN	-	Supra-Chiasmatic Nucleus
ZT	-	Zeitgeber Time
CPCSEA	-	Committee for the Purpose of Control and Supervision of Experiments on Animals
IAEC	-	Institutional Animal Ethical Committee
TMRM	-	Tetra-methyl rhodamine methyl ester
NAC- N	-	acetylcysteine
Mel	-	Melatonin
BMAL1	-	Brain and muscle ARNT-Like 1
HPLC	-	High-performance liquid chromatography
ANOVA	-	One-way analysis of variance
SDH	-	Succinate dehydrogenase
CLOCK	-	Circadian locomotor output cycles kaput

LIST OF FIGURES

Figure 1.1 Proposed hypothesis.....	16
Figure 2.1 Study design	22
Figure 2.2 Wheel running locomotor activity rhythm in mice	30
Figure 2.3 Temporal variations in mitochondrial respiration in SCN.....	32
Figure 2.3.C Daily rhythms in respiratory control rate (RCR).....	33
Figure 2.4 Daily fluctuation in mitochondrial copy number over 24 h.....	36
Figure 2.4 C Daily rhythms in mitochondrial abundance.....	37
Figure 2.4 D correlation analysis of RCR and Mito DNA.....	38
Figure 2.5 Relative mRNA expressions of core clock genes	39
Figure 2.6 Plasma corticosterone levels.....	41
Figure 2.6 C Daily rhythms of plasma CORT levels.....	41
Figure 3.1 Study design.....	53
Figure 3.2 (a) Respiratory control ratio (RCR) and (b) states in SCN mitochondria.....	60
Figure 3.3 (a) GSH and (b) Glutamate level in the SCN.....	61
Figure 3.4 ATP level in the SCN.....	62
Figure 3.5 Blood plasma melatonin level.....	62
Figure 3.6 Plasma CORT level.....	63
Figure 3.7 Relative mRNA expressions of core clock genes in the SCN.....	65
Figure 4.1 Study design.....	73
Figure 4.2 Body weight of mice.....	79
Figure 4.3 Feed intake of mice/week.....	80
Figure 4.4 Respiratory control ratio (RCR)/states.....	81
Figure 4.5 SDH Staining images and bar graph	82
Figure 4.6 GSH level in the liver.....	83
Figure 4.7 ATP level in the liver.....	84
Figure 4.8 Blood plasma glucose level (a), triglyceride (b), total cholesterol (c), and HDL.....	85
Figure 4.9 Plasma Leptin (a) and Ghrelin (b) level.....	86
Figure 5.1 Study design.....	95
Figure 5.2 Effect of 5% and 10% DMSO diluents on mitochondrial bioenergetics.....	100

Figure 5.3 Effect of Q10, RSV and MET on respiratory control ratio (RCR).....	101
Figure 5.4 Respiratory control ratio (RCR)	102
Figure 5.5 Complexes enzyme activity in isolated brain mitochondria.....	104
Figure 5.6 Mitochondrial membrane potential (MMP)	105
Figure 6.1 Summary and conclusion of the study.....	112

LIST OF TABLES

Table 2.1 List of the primers	26
Table: 2.2 Different mitochondrial respiration states.....	34
Table 3.1 List of the primers.....	58

PREFACE

The thesis research work entitled “Pharmacological intervention in dim light induced changes in circadian rhythm.” assessed the effect of continuous dim light on the rhythmicity of mitochondrial function in the superchiasmatic nuclei (SCN). Further, we evaluated the role of mitochondria in normal circadian rhythm (light dark; LD 12:12 h) and disrupted circadian rhythm (continuous dim light; LL 24:00 h). Further, first time we are showing there is a rhythmic pattern in mitochondrial bioenergetics and mitochondrial DNA in LD condition over the 24-h in along with corticosterone and the rhythmicity was lost in the LL condition. Therefore, we sought to the effect of mitochondrial modulator N-acetylcysteine (NAC) on dim light induced mitochondrial changes in central and peripheral tissue clock. Moreover, we evaluated the NAC effect on different circadian clock genes, endogenous melatonin and hunger hormone leptin and ghrelin which was attenuated at different doses with NAC in mice exposed to dim light. Furthermore, there are several pharmacological agents like metformin that are promising to have a mitochondrial potential or inhibitory effect there is no screening method to test their activity on mitochondria. Hence, we have developed a robust economic *ex-vivo* method for mitochondrial bioenergetics using mitochondrial modulators.

The whole work has been compiled into six chapters: **Chapter 1** introduces the topic and its importance. **Chapter 2** Effect of normal and disrupted circadian rhythm on the mitochondria. **Chapter 3** Effect of N-acetylcysteine on mitochondria dysfunction induced by disrupted circadian rhythm in central clock. **Chapter 4** documents the effect of N-acetylcysteine on mitochondria dysfunction induced by disrupted circadian rhythm in peripheral clock. **Chapter 5** Novel *Ex-vivo* method development for the evaluation of mitochondrial modulators and **Chapter 6** summarizes the entire study with the conclusion and important outcomes.

