Chapter 4

4. MATERIALS AND METHODS

4.1 Study Overview

This research employed a dual-model approach to investigate chloramphenicol-induced mitochondrial liver toxicity and evaluate the hepatoprotective potential of antioxidants Astaxanthin and Quercetin. The study consisted of:

- *In-vitro* experiments using HepG2 cells to assess mitochondrial toxicity, reactive oxygen species (ROS), and gene expression profiles.
- *In-vivo* experiments in Wistar rats to assess biochemical markers of oxidative stress, specifically glutathione (GSH) and nitric oxide (NO), following chloramphenicol exposure and antioxidant intervention.

All experimental procedures adhered to standard protocols and ethical guidelines.

4.2 *In-Vitro* Methods

4.2.1 Materials

- Cell line: Human hepatocellular carcinoma cells (HepG2; ATCC)
- Chemicals: Chloramphenicol, Astaxanthin, Quercetin (Sigma-Aldrich)
- Assay Kits: CellTiter-Glo® Luminescent Cell Viability Assay (Promega) , ROS-Glo™ H O Assay Kit (Promega)
- Media & Reagents: DMEM (glucose and galactose), FBS, antibiotics, pyruvate, L-glutamine (HiMedia)

4.2.2 Cell Culture and Treatment

Cell maintenance: HepG2 cells were maintained in DMEM (glucose or galactose) with 10% FBS, antibiotics, and supplements. Incubated at 37°C in 5% CO .

Drug preparation: Chloramphenicol stock (600 mM in DMSO) was diluted to final concentrations (3–3000 µM). Astaxanthin (5–15 µM) and Quercetin (10–30 µM) were prepared similarly.

Control groups: Untreated and 0.5% DMSO vehicle controls were used for baseline comparison.

4.2.3 ATP-Based Mitochondrial Toxicity Assay

HepG2 cells were plated in collagen I-coated 96-well plates at 10 cells/mL,After overnight incubation in galactose media, cells were exposed to chloramphenicol for 6 days, with or without antioxidants. ATP content was measured using the CellTiter-Glo® assay. Luminescence was recorded with a microplate reader.

4.2.4 ROS Assessment

ROS levels were measured using the ROS-Glo™ H O assay. Cells were treated with chloramphenicol (3000 μM), Astaxanthin (10 μM), or Quercetin (25 μM) for 48 h. Rotenone was used as a positive control for ROS induction. Results were expressed as relative luminescence units (RLU).

4.2.5 Gene Expression Profiling

Total RNA was isolated using Trizol reagent. The reverse transcription was done using Thermo Fisher cDNA synthesis kit. Quantitative PCR (qPCR) was performed with SYBR Green on an Applied Biosystems 7500 Fast system. Target genes: SOD2, NRF1, SURF1, TFAM, and UCP2; reference gene: RPLP1.Expression changes were calculated using the 2 ΔΔCT method. Target gene detail for gene expression study and Primer sequences and accession numbers is provided in Table 3 and 4.

NCBI Reference Sequence	Gene Symbol	Gene Name	Biological Function
NM_001280787.1	SURF1	Surfeit locus protein 1	This gene encodes a protein localized to the inner mitochondrial membrane and thought to be involved in the biogenesis of the cytochrome c oxidase complex.
NC_000006.12	SOD2	Superoxide dismutase 2	This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen. In case of increased oxidative stress, the expression of SOD2, which is involved in combating oxidative stress, might be upregulated as a cellular response to counteract ROS.
NC_000007.14	NRF1	Nuclear respiratory	This gene encodes a protein that homodimerizes and functions as a transcription factor which

		factor 1	activates the expression of some key metabolic genes regulating cellular growth and nuclear genes required for respiration, heme biosynthesis, and mitochondrial DNA transcription and replication
NC_000010.11	TFAM	Transcription factor A, mitochondrial	This gene encodes a key mitochondrial transcription factor containing two high mobility group motifs. The encoded protein also functions in mitochondrial DNA replication and repair.
NC_000011.10	UCP2	Uncoupling protein 2	UCPs may impact the mitochondrial membrane potential in mammalian cells.

Table 3A: Target gene detail for gene expression study

Gene	Mitochondrial Function	Involvement in DILI
SOD2	ROS detoxification	Protects against oxidative mitochondrial injury
NRF1	Regulates mitochondrial biogenesis	Maintains mitochondrial transcription and function
SURF1	Complex IV assembly	Essential for ETC function and ATP production
TFAM	mtDNA transcription & replication	Key for mitochondrial gene expression
UCP2	Modulates membrane potential & ROS	Balances ATP synthesis and oxidative stress
RPLP1	Ribosomal protein (reference gene)	Stable control gene for mRNA normalization

Table 3B: Summary Table of Genes selected for study

Gene symbol	Primer sequence	Accession number
SURF1	Forward: 5'GTGGTCACTCCCTTCCACTG3' Reverse: 5′ TCTGCCGGGTTTCAGGATTC3′	NM_001280787

SOD2	Forward: 5'CGGTAGCACCAGCACTAGC3' Reverse: 5′GATACCCCAAAACCGGAGCC3'	NM_001024465.3
NRF1	Forward: 5''ACACAGAAAAGGTGCTCAAAGG3′ Reverse: 5''TGTTCGGTTTGGGTCACTCC3′	NM_001040110.2
TFAM	Forward: 5′ GCGGGTTCCAGTTGTGATTG 3′ Reverse: 5 CACATGCTTCGGAGAAACGC ′3′	NM_001270782.2
UCP2	Forward: 5' GGGATTGACTGTCCACGCTC 3' Reverse: 5 ATACTATGTGTCCGAGCCGC3'	NM_003355.3
RPLP	Forward: 5' CTCACTTCATCCGGCGACTA 3' Reverse: 5' GCCAGGGCCGTGACTGT 3'	NM_213725.2

Table 4: Sequences of primers for real-time PCR

4.3 Detail of Mitochondrial-Related Genes Selected for the research:

1. SOD2 (Superoxide Dismutase 2, mitochondrial)

- **Function:** SOD2 encodes the mitochondrial manganese-dependent superoxide dismutase enzyme.
- **Role in Mitochondria:**
  - Catalyzes the dismutation of superoxide radicals (O •–) into hydrogen peroxide (H O) and molecular oxygen.
  - Localized in the mitochondrial matrix, it is critical for antioxidant defense and redox homeostasis in mitochondria.
  - Protects mitochondrial DNA (mtDNA), enzymes, and membranes from oxidative damage.
- **Relevance to DILI:**
  - Impairment or downregulation of SOD2 leads to mitochondrial oxidative stress, a key driver in DILI and apoptosis.

2. NRF1 (Nuclear Respiratory Factor 1)

- **Function:** NRF1 is a transcription factor that regulates genes involved in mitochondrial biogenesis and function.
- **Role in Mitochondria:**

- Activates transcription of key mitochondrial proteins, including those involved in respiratory chain complexes, heme biosynthesis, and mitochondrial DNA transcription/replication.
- Stimulates expression of TFAM, a critical factor for mtDNA transcription and maintenance.
- Orchestrates mitochondrial biogenesis and respiratory capacity.

- **Relevance to DILI:**
    - Downregulation disrupts mitochondrial homeostasis, energy metabolism, and adaptive responses to drug-induced stress.

3. SURF1 (Surfeit Locus Protein 1)

- **Function:** SURF1 encodes a mitochondrial inner membrane protein involved in assembly of cytochrome c oxidase (complex IV of the respiratory chain).
- **Role in Mitochondria:**
- Facilitates proper assembly and function of Complex IV, the terminal enzyme of the electron transport chain (ETC).
- Essential for efficient electron transfer and ATP production.

- **Relevance to DILI:**

- SURF1 dysfunction impairs OXPHOS, leading to energy deficiency and increased ROS.
- Mutations are linked to Leigh syndrome, a mitochondrial encephalopathy—highlighting its critical role.

4. TFAM (Mitochondrial Transcription Factor A)

- **Function:** TFAM is a DNA-binding protein required for transcription and replication of mitochondrial DNA.
- Binds mtDNA and initiates transcription by recruiting the mitochondrial RNA polymerase complex.
- Stabilizes mtDNA and prevents its degradation.

- Acts as a key regulator of mitochondrial biogenesis and maintenance of mitochondrial gene expression.
- **Relevance to DILI:**
    - Reduced TFAM expression compromises mitochondrial replication and respiratory function, sensitizing hepatocytes to toxic injury.

5. UCP2 (Uncoupling Protein 2)

- **Function:** UCP2 belongs to the mitochondrial anion carrier protein family and is involved in proton leak across the inner mitochondrial membrane.
- **Role in Mitochondria:**
    - Modulates the proton gradient, reducing mitochondrial membrane potential and reactive oxygen species (ROS) generation.
    - Acts as a protective mechanism during oxidative stress by uncoupling OXPHOS.
    - Also implicated in metabolic reprogramming and regulation of insulin secretion and fatty acid metabolism.

Relevance to DILI:

- Elevated in response to mitochondrial oxidative stress; however, excessive uncoupling can reduce ATP synthesis.
- Balancing UCP2 expression is crucial for maintaining redox and energy homeostasis during drug exposure.

4.4 Rationale for Using RPLP1 as a Reference Gene

RPLP1 (Ribosomal Protein, Large, P1)

Function:

- Encodes a ribosomal protein that is a component of the 60S subunit of the ribosome.
- Involved in translation elongation and protein synthesis.
- Maintains structural integrity of ribosomes and ensures efficient mRNA translation.

Why RPLP1 is a Suitable Reference Gene in Mitochondrial Studies:

1. **Stable Expression:**

RPLP1 exhibits low variability across tissues and experimental conditions, including drug-treated hepatic cells.

2. Non-mitochondrial Function:

Its function is housekeeping and unrelated to mitochondrial pathways, making it a neutral comparator when studying mitochondrial gene expression.

3. Validated in Hepatic Models:

RPLP1 has been shown to maintain stable expression in HepG2 cells and liver tissues, including under oxidative and drug-induced stress, making it suitable for normalization in RT-PCR studies.

4. Less Susceptible to Regulation by Stress:

Unlike ACTB or GAPDH, which may be modulated under stress conditions, RPLP1 expression remains consistent, providing reliable quantification in qPCR.

4.5 *In Vivo* Methods

This preclinical, randomized, interventional study was conducted after obtaining required ethical approval from IAEC (ZRC/DMPK/BP/056/04-2K23) at the Zydus Research Centre, Gujarat, India, from May to July 2023. All the experiments were conducted in accordance with the guidelines and regulations set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and approved by the Institutional Animal Ethics Committee (IAEC). Animals and Grouping.

Subjects: 24 male Wistar rats (180–200g, 6–8 weeks old)

Housing: 12h light/dark cycle, 22 ± 2°C, 60–70% humidity

- **Group I:** Control Group (n=6): No treatment (normal saline).
- **Group II:** Chloramphenicol Group (n=6): Treated with Chloramphenicol (25 mg/kg body weight, intraperitoneally) for 14 days
- **Group III:** Chloramphenicol + Astaxanthin Group (n=6): Treated with Chloramphenicol (25 mg/kg body weight, intraperitoneally) for 14 days, followed by Astaxanthin (20 mg/kg body weight, orally) for 14 days
- **Group IV:** Chloramphenicol + Quercetin Group (n=6): Treated with Chloramphenicol (25 mg/kg body weight, intraperitoneally) for 14 days, followed by Quercetin (30 mg/kg body weight, orally) for 14 days

4.5.1 Biochemical Assays

Sample collection: Blood samples were drawn via retro-orbital plexus on Day 0, Day 15, and Day 30.

GSH assay: Based on enzymatic recycling with DTNB (R&D **Systems,** 7511-100-K); absorbance at 405 nm.

NO assay: Total nitrite levels estimated using Griess Reagents (R&D **Systems,** KGE001); absorbance at 540 nm.

4.6 Statistical Analysis

4.6.1 *In-Vitro* Analysis:

All experiments were conducted in **triplicates** on three independent days (n = 3 biological replicates), with three technical replicates per treatment group in each experiment. Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism version 8.0. Group comparisons were analyzed using one-way ANOVA, followed by Dunnett's multiple comparisons test for ROS and ATP assays, and Bonferroni's post hoc test for gene expression studies. A p-value < 0.05 was considered statistically significant.

4.6.2 *In-Vivo* Analysis:

All statistical analyses were performed using GraphPad Prism software, version 8.0.2 (GraphPad Software, San Diego, CA, USA). Statistical comparisons between groups were conducted using one-way analysis of variance (ANOVA), followed by Sidak's multiple comparisons test for multiple comparisons. The significance level of $p < 0.05$ was considered statistically significant. Data are presented as mean ± standard deviation (SD) unless otherwise specified.

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