Chapter 6

6. DISCUSSION

Drug-induced liver injury (DILI) remains a prominent cause of acute liver failure and drug withdrawals worldwide. Among its various mechanisms, **mitochondrial dysfunction (MD)** has increasingly been recognized as a central driver of hepatocellular damage, particularly due to the liver's reliance on oxidative phosphorylation (OXPHOS) for maintaining energy homeostasis. This study was designed to investigate the hepatotoxic potential of **chloramphenicol**, a historically significant yet hepatotoxic antibiotic, with a mechanistic focus on mitochondrial impairment. Additionally, the research aimed to explore the **mitochondria-targeted protective efficacy** of two well-characterized natural antioxidants **Astaxanthin** and **Quercetin** in both in-vitro and in-vivo experimental models.

Chloramphenicol-Induced Mitochondrial Toxicity

Chloramphenicol exerts its bacteriostatic action by binding to the 50S ribosomal subunit in prokaryotes, inhibiting peptide bond formation during translation. However, it also binds the **55S mitochondrial ribosome** in eukaryotic cells particularly hepatocytes thereby inhibiting mitochondrial protein synthesis. This disruption compromises **complex I–IV assembly**, **impairs ATP synthesis**, and promotes **electron leakage**, resulting in the **generation of reactive oxygen species (ROS)** [118,119]. In our **in-vitro studies**, HepG2 cells adapted to galactose a condition that enforces mitochondrial dependence exhibited **marked ATP depletion** upon chloramphenicol exposure. The observed **IC fold change exceeding 1.5** (glucose vs. galactose media) aligns with the FDA's accepted threshold for indicating **mitochondrial-specific toxicity** [158].

Moreover, chloramphenicol exposure significantly elevated **intracellular ROS**, highlighting the role of oxidative stress as a downstream effector of mitochondrial injury. This observation is in line with previous studies showing chloramphenicol-induced **electron transport chain (ETC) disruption**, excessive **superoxide formation**, and activation of **mitochondrial apoptotic pathways** [159]. These findings substantiate the drug's mitotoxic profile and validate its use as a model agent in DILI research.

Antioxidant Protection by Astaxanthin and Quercetin

Astaxanthin and Quercetin were selected based on their **antioxidative, anti-inflammatory, and mitochondrial-protective profiles**. Treatment with either compound significantly mitigated the toxic effects of chloramphenicol. Astaxanthin's **lipophilic structure** allows integration into the mitochondrial membrane, where it directly **scavenges singlet oxygen and superoxide**, stabilizes membrane potential, and inhibits the **release of cytochrome c**, thereby preventing apoptosis [160]. Quercetin, by contrast, is known to **activate Nrf2 signaling**, upregulate **phase II detoxifying enzymes**, and enhance intracellular GSH synthesis [160–162].

Our study revealed that both compounds **restored ATP levels**, **reduced ROS**, and **improved mitochondrial gene expression patterns**. Notably, **Quercetin** was slightly more effective in **restoring GSH levels** in vivo, possibly due to its influence on the **glutathione biosynthetic pathway**, while **Astaxanthin** exerted more pronounced effects on **ROS suppression** at the mitochondrial level.

Mitochondrial Gene Expression: Insights into Mechanisms

Analysis of mitochondrial gene expression provided mechanistic insights into the differential responses:

- **SOD2 (Superoxide Dismutase 2)** and **UCP2 (Uncoupling Protein 2)** were **upregulated** in chloramphenicol-only groups, representing a compensatory response to mitochondrial ROS accumulation [161,162].
- **NRF1 (Nuclear Respiratory Factor 1)** upregulation indicates activation of **mitochondrial biogenesis** pathways, reflecting an adaptive effort to maintain mitochondrial mass and function [163].
- **TFAM (Transcription Factor A, Mitochondrial)** and **SURF1**, critical for **mtDNA transcription and complex IV assembly**, were **downregulated**, indicating loss of mitochondrial transcriptional integrity and ETC disruption [164,165].

This gene expression signature paints a picture of **mitochondrial stress response** where the cell attempts to restore redox and bioenergetic balance, but fails to do so effectively under sustained chloramphenicol insult.

Comparative Efficacy and Novelty

Compared to prior studies, our research stands out for its **targeted evaluation of antioxidant effects specifically in a chloramphenicol-induced mitotoxic model**. Previous studies often generalized the antioxidant benefits of these compounds in diverse liver injury models. Here, we provide **evidence-based insights** into how **Astaxanthin and Quercetin directly modulate mitochondrial gene expression**, oxidative balance, and cellular energy homeostasis in the context of antibiotic-induced mitochondrial stress.

Key contributions of this study include:

- **Targeted Therapeutic Application**: This work specifically identifies Astaxanthin and Quercetin as viable agents against **antibiotic-induced mitotoxicity**, moving beyond generic antioxidant claims.
- **Mechanistic Elucidation**: By evaluating expression levels of mitochondrial genes, this research lays the foundation for future mechanistic studies focusing on **mitochondrial transcriptional and post-translational regulation**.
- **Benchmarking Efficacy**: The comparative analysis of both antioxidants offers practical guidance for selecting compounds based on desired endpointse.g., ROS suppression vs. GSH replenishment.
- **Potential for Broader Application**: The findings open avenues for investigating other naturally occurring or synthetic antioxidants with **mitochondria-targeted activity**, especially those affecting **mitochondrial dynamics**, such as **fusion, fission, and mitophagy**.

In-Vivo Validation and Translational Relevance

The **in-vivo component** of our study not only validated the in-vitro findings but also offered **systemic insights** into chloramphenicol-induced liver toxicity. Notably, the **GSH depletion and NO elevation** seen in Wistar rats were consistent with classical indicators of **oxidative and nitrosative stress**. Co-treatment with antioxidants reversed these biochemical alterations. **Quercetin** demonstrated marginal superiority in **replenishing GSH**, while **Astaxanthin** excelled in reducing hepatic ROS and preserving mitochondrial function.

These differences may be attributed to **pharmacokinetic variability**, including **bioavailability, intracellular distribution, and metabolite stability**. Quercetin primarily acts through **cytosolic antioxidant pathways**, whereas Astaxanthin preferentially accumulates in **mitochondrial membranes**, suggesting **complementary modes of action** [156].

Our findings also resonate with previous reports: **Liu et al.** showed that **nanoliposomal Quercetin** attenuates acetaminophen-induced liver injury [150], while **Yamashita et al.** demonstrated that **Astaxanthin reduces mitochondrial swelling and cytochrome c release**, thus preventing hepatocyte apoptosis [151].

Comparison with Other Antioxidants

Antioxidants like **N-acetylcysteine (NAC)**, **resveratrol**, and **vitamin E** have also demonstrated hepatoprotective effects [147–149]. However, what distinguishes Astaxanthin and Quercetin is their **specific modulation of mitochondrial genes** and **dual action** on both **oxidative stress and energy metabolism**. Unlike general antioxidants that work primarily via redox scavenging, these compounds **intervene at the level of gene expression, mitochondrial signaling, and biogenesis**, offering a more **sustained and targeted therapeutic effect**.

Significance in the Context of Regulatory Science and Drug Safety

This study holds particular importance for **regulatory toxicology**. Many adverse drug reactions leading to DILI are **missed in preclinical screens** due to the limited sensitivity of traditional hepatotoxicity models to detect **mitochondrial liabilities**. Standard glucose-based HepG2 models often mask mitochondrial-specific toxicities because these cells preferentially utilize glycolysis over oxidative phosphorylation. The **galactose-conditioning strategy** employed in this thesis enhances mitochondrial reliance, making it a **sensitive and reliable model** to screen for mitochondrial dysfunction a technique gaining increasing traction in pharmaceutical safety testing.

Furthermore, regulatory bodies like the **FDA**, **EMA**, and **ICH** are gradually recognizing the importance of **integrating mitochondrial toxicity assays** into safety pharmacology frameworks. This research contributes directly to that paradigm shift by showcasing how in-vitro mitochondrial stress models can align with in-vivo findings, thereby **bridging the gap between bench and bedside**.

Immuno-Mitochondrial Interactions and Inflammation

An emerging dimension in DILI research is the **interaction between mitochondria and immune responses**. Damaged mitochondria release **mitochondrial DNA (mtDNA)**, **formyl peptides**, and **ROS**, which act as **mitochondrial damage-associated molecular patterns (mtDAMPs)**. These mtDAMPs activate **Toll-like receptors (TLRs)** and **NOD-like receptors (NLRs)**, including **NLRP3 inflammasome**, promoting **Kupffer cell activation**, cytokine secretion, and **sterile inflammation**.

Though not the primary focus of this study, the potential of **Astaxanthin and Quercetin** to modulate these pathways warrants exploration. Both antioxidants have been shown in other models to suppress **NF-κB** signaling and reduce **IL-1β and TNF-α**, suggesting a broader immunomodulatory role that could further mitigate hepatic inflammation in DILI.

Mitochondrial Dynamics and Quality Control

The regulation of mitochondrial health involves **fusion, fission, biogenesis, and mitophagy** collectively termed **mitochondrial dynamics**. Disruption of these processes can lead to **mitochondrial fragmentation**, accumulation of damaged mitochondria, and apoptosis. Preliminary studies suggest that chloramphenicol may impair mitochondrial dynamics, although this was not directly assessed in the current work. Future investigations could examine whether Astaxanthin and Quercetin influence these processes by modulating key proteins like **OPA1**, **MFN1/2**, **DRP1**, and **PINK1/Parkin**.

Such insights could pave the way for antioxidant therapies that not only quench ROS but also **restore mitochondrial integrity** via quality control mechanisms.

Emerging Biomarkers and Diagnostic Potential

Traditional biomarkers like **ALT, AST, and bilirubin** lack sensitivity and specificity in detecting mitochondrial injury. Newer biomarkers, including **GLDH (glutamate dehydrogenase)**, **circulating mtDNA**, and **miR-122**, have shown promise in identifying **early-stage mitochondrial damage**. While these markers were not assessed in this study, they represent **valuable adjuncts** for future translational validation, particularly in clinical trials evaluating antioxidant therapy in DILI patients.

Relevance in Global Health and Low-Resource Settings

Chloramphenicol is still used in **resource-constrained healthcare systems**, particularly in treating infections like **typhoid fever** and **rickettsioses**. Given its affordability and broad-spectrum efficacy, removing chloramphenicol from essential drug lists could jeopardize infectious disease management in **low- and middle-income countries (LMICs)**. Therefore, the **co-administration of hepatoprotective antioxidants** provides a viable strategy to preserve its clinical utility while minimizing harm.

This approach resonates with the **rational drug use** principles promoted by the **WHO** and supports equitable access to safe medications.

Limitations and Future Directions

While the current findings are compelling, several limitations must be acknowledged:

- The **dose optimization** of antioxidants for clinical translation remains unaddressed.
- **Histopathological liver assessments**, including electron microscopy, could further validate mitochondrial integrity.
- The **time-course expression** of mitochondrial and inflammatory genes post-chloramphenicol exposure was not assessed.
- The **interactions between chloramphenicol metabolites and mitochondria** remain unclear and could be explored using metabolomics.

Building upon these limitations, future studies could also explore **combination antioxidant therapy**, pharmacokinetics in humanized models, and **gene-editing techniques** (e.g., CRISPR-Cas9) to assess the direct role of mitochondrial genes in DILI pathogenesis.

Conclusion (Integrated Summary)

This research **comprehensively evaluated the hepatotoxic effects of chloramphenicol** through the mechanistic lens of mitochondrial dysfunction and demonstrated the **hepatoprotective efficacy of Astaxanthin and Quercetin**. Through a dual-model approach using **galactose-adapted HepG2 cells** and **Wistar rats**, the study established a robust connection between chloramphenicol-induced oxidative stress, **mitochondrial gene dysregulation**, and **ATP depletion**.

Key outcomes include:

- Demonstration of **mitochondria-dependent toxicity** in chloramphenicol-treated HepG2 cells, especially under forced OXPHOS conditions.
- **In-vivo validation** showing oxidative stress markers (↓GSH, ↑NO) mirroring in-vitro mitochondrial damage.
- Restoration of mitochondrial function, gene expression normalization, and ROS reduction upon **antioxidant intervention**.
- **Differential efficacy** of Quercetin (higher GSH recovery) and Astaxanthin (stronger ROS suppression), suggesting distinct therapeutic advantages.

The study thus highlights the **utility of tailored in-vitro models** for early identification of mitotoxic drugs and positions **Astaxanthin and Quercetin** as **viable adjunct therapies** for reducing antibiotic-induced hepatotoxicity. These findings also pave the way for **regulatory refinement**, **translational biomarker development**, and **personalized antioxidant strategies** in managing DILI.