Virtual Experiments Falsify a Prevailing Mechanistic Explanation for Acetaminophen Induced Liver Injury and Enable Discovery of Plausible Alternative Mechanisms

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Acetaminophen induced liver injury is a multifaceted phenomenon potentially causing liver failure. A precondition to developing strategies that avoid, disrupt, and/or reverse liver damage is a reliable causal cascade description. Yet even after three decades of intense investigation, a cause-effect relationship remains elusive. The weight of the evidence supports this hypothesis: zonation (spatial heterogeneity) within hepatic lobules of NAPQI formation (the reactive metabolite) can account for necrosis occurring first adjacent to the lobule’s central vein (Mechanism Composite 1). However, the hypothesis cannot be tested directly in mice because doing so requires sequential intracellular measurements at different locations within hepatic lobules, which is currently infeasible.

We challenged and refuted—falsified—Mechanism Composite 1 using virtual experiments with concrete mechanisms. To date, objects of acetaminophen toxicity experiments have included animal models, organotypic hepatocyte cultures, and organ-on-chip systems. But controlling key variables during such experiments has proven problematic. Consequently, results have fallen short of expectations. We experimented on virtual mice containing a concretized biomimetic liver lobule model of Mechanism Composite 1, which had already achieved multiple multiscale qualitative and quantitative validation targets. Virtual hepatocyte objects can exhibit lobule-location dependent behaviors. We first achieved quantitative validation targets for acetaminophen clearance and metabolism. We then implemented and verified a concrete, parsimonious version of Mechanism Composite 1 and imposed several evidence-based constraints. A parameter space search failed to produce a parameterization that could achieve the key validation target: necrosis first occurs adjacent to the lobule’s central vein. We posited that at least one additional feature must also exhibit zonation to achieve the validation target. We developed and challenged two alternative hypotheses: either amount of GSH (Mechanism Composite 2) or amount of mitochondrial damage (Mechanism Composite 3) must exhibit zonation in addition to NAPQI formation. Alternative mechanisms were evaluated based on their composed behavior (phenotype). Mechanism composites can be selected (for or against) based on whole or decomposed pattern/phenotype. Both hypotheses were falsified.

We then challenged a fourth hypothesis: zonation in amount of available GSH and mitochondrial damage repair (in addition to NAPQI formation) are required to achieve the validation target. That mechanism achieved the validation target.

In silico outcomes are multiscale consequences of two location-dependent counteracting mechanisms. We hypothesize that corresponding mechanisms occur within mouse lobules upon exposure to a toxic APAP dose: two types of intracellular injury initiated by NAPQI: mitochondrial (mitoDamage) & non-mitochondrial (nonMD) damage. The pace of repair of (recovery from) mitochondrial injury determines if Necrosis is triggered or not.
**BACKGROUND**

**Multiorgan Biological Features**

- Mechanisms of acetaminophen (APAP) hepatotoxicity have been under intensive investigation for several decades.
- APAP overdose causes multiple interrelated molecular level events.
- Two critical relative importance in reaching hepatotoxicity is now more evident. That is, transient, liver-related damage is believed to be a primary determinant.
- Mitochondrial dysfunction (mitoDamage) and cell death are consequences.
- In vitro studies contrasted with in vivo studies show the need for both macroscopic and mesoscopic understanding of the problem.
- APAP hepatotoxicity incorporates dual mechanisms. First, is destruction and motility of liver mitochondria. Second, is a cell death process.

**APAP Hepatotoxicity**

- This work focuses on the first 12 hours following a toxic dose.

**The Promoting Explanatory Hypothesis**

- The weight of the evidence suggests the hypothesis: In the evolution of NAPQI formation, a key event is the activation of cytochrome C, and then damage in the PV (portal vein) direction.

**Science Demands**

- What do we mean by explanatory hypotheses?
- What are the criteria that define a hypothesis?
- How are hypotheses developed and tested?
- What are the limitations of current research?

- Challenges of an explanatory hypothesis includes:
  - Understanding the complex biological systems involved in liver injury.
  - Developing models that can predict outcomes.

- Our Solution
  - Concretize and experiment on competing plausible mechanisms.
  - Analyze the data collected.
  - Develop a new hypothesis.

**In Vivo Liver**

- Mouse Relationships
  - Hepatocyte function is dependent on location within the liver.
  - The liver is composed of individual lobules.
  - The lobules contain individual cells that have different functions.

**In Silico Liver**

- About the Iterative Refinement Protocol
  - The current protocol includes the use of Monte Carlo simulation.
  - Only composite mechanisms can be evaluated (falsified).
  - Core Method
    - Validation targets: changes in GSH and ROS levels, NAPQI formation.
    - In Silico Liver
      - Virtual experiments falsify a prevailing mechanism.
      - Concretization and experiment on competing plausible mechanisms.
      - Achieves better understanding of liver injury.

**METHODS**

- New Validation Targets
  - Mortality
  - NAPQI formation
  - Mitochondrial dysfunction
  - DNA fragmentation
  - Viable hepatocytes
  - NAC treatment
  - NAC treatment
  - GSH depletion
  - ROS formation

- New Insight, New Hypothesis
  - DNA damage
  - Mitochondrial dysfunction
  - Cell death

- Methodological Take Home Lessons
  - Virtual experiments offer unique opportunities to test hypotheses and explore mechanisms.
  - In vivo and in vitro models are complementary.
  - GSH depletion and ROS formation play key roles in liver injury.

**Mechanistic Inferences**

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