Hepatotoxicity Testing of Acetaminophen in Two- and Three-Dimensional Rat Hepatocyte Cultures

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Abstract

Many pharmaceutical drugs are unable to pass clinical trials due to unpredicted liver damage. Animal models are currently used for drug testing but are expensive and often inaccurate. Monolayer (2D) cell culture models have been utilized due to lower cost, but have proven less reliable because they are unable to preserve the functionality of the liver \textit{in vitro}. This study aimed to establish a more accurate and inexpensive pharmaceutical testing model that has high reproducibility. Rat hepatocytes were grown in both two-dimensional (2D) and three-dimensional (3D) cultures. Recent studies have shown promising results for 3D cultures, which increase cell to cell interactions and better preserve cell function. 3D cultures were grown using a mold designed and manufactured in previous REU research. Cells were treated with acetaminophen and analyzed for cytotoxicity using a Neutral Red Dye. Cell functionality was then tested by quantification of albumin secretion using an Enzyme-Linked Immunosorbent Assay (ELISA). Both 2D and 3D cultures showed a decline in albumin production within the 0.00-2.25 mM acetaminophen concentration range after 48 hours of treatment. After 72 hours of treatment, 3D cultures showed an 81.2\% decline in albumin concentration with only 0.15 mM APAP while 2D cultures increased albumin concentrations by 0.63\%. This shows that when cells are grown in cultures more similar to the environment of the liver, they have a higher sensitivity to hepatotoxicity.  

Keywords: Hepatotoxicity, Acetaminophen, H35 Rat Hepatocyte, Three-Dimensional Culture

1. Introduction

It is estimated that over one thousand drugs available to the public have caused liver disease\(^1\). Despite extensive clinical trials required before a drug can enter the market, drug-induced liver injury is cited as the most common cause of acute liver failure in the United States\(^2\). The current “gold standard” for pharmaceutical testing is animal studies. Animals are expensive to obtain and cannot be used by small-scale labs. These studies are also inaccurate, failing to predict about 50\% of drug-induced liver injury\(^2\). New models are needed in order to lower the cost of testing as well as make them more available to smaller labs.

1.1 Hepatotoxicity

Hepatocytes are cells comprising 80\% of the liver, the second largest organ in the human body\(^3\). The liver is responsible for many functions, including the production of bile and the secretion of waste; the most important function being the filtration of blood. No blood can exist in the bloodstream without passing through the liver.
The liver is therefore a major organ for drug metabolism because it is able to filter out any toxins associated with the drug. Hepatotoxicity is defined as any liver injury associated with overexposure to toxins\textsuperscript{4}, resulting in a loss of function of the hepatocytes.

1.2 Acetaminophen
The most common drug associated with hepatotoxicity is acetaminophen (also known as APAP or paracetamol). Acetaminophen is the active ingredient in common pain/fever reducers and is the cause of 50% of all liver transplants in the United States\textsuperscript{2}. As APAP enters the liver it is readily metabolized to $N$-acetyl-$p$-benzoquinoneimine (NAPQI). This metabolite is toxic, but at recommended therapeutic doses (about 20 mg/kg\textsuperscript{5}) it can be excreted in the urea with no damage. At high concentrations the liver is unable to filter NAPQI and it begins to bind to proteins and DNA within the bloodstream, resulting in hepatotoxicity\textsuperscript{5}.

1.2.1 Model Drug
Acetaminophen has been a common over-the-counter prescription since the mid-1900s. Because of its long history and prevalent hepatotoxicity, it has been widely studied and is easily available to any laboratory. These factors make APAP a good model for developing a clinical drug test. The reactive metabolite of acetaminophen is known, but for most drugs it is unpredictable. 85% of drugs that are tested through clinical trials are found to have reactive metabolites\textsuperscript{3}. Perfecting a model to detect NAPQI effects within hepatocytes can then be applied to newly developed drugs.

1.3 Current Methods
Drug testing models have moved away from in vivo animal studies and are seeing a focus on in vitro cultures. Studies have cultured hepatocytes in two-dimensional (2D) monolayers but these cultures have proven unsuccessful in predicting hepatotoxicity. Monolayered cells are unable to preserve their functions outside of the liver and have shown a decrease in function without any form of drug treatment\textsuperscript{6}. Other studies have shown a higher tolerance for APAP in the monolayer than in the body. The hepatocytes were unable to metabolize APAP to NAPQI, therefore reducing the toxic effects of APAP\textsuperscript{7}. This leads to a false conclusion of a higher drug tolerance. In order to preserve the functionality of hepatocytes, methods have been created to mimic the environment of the liver in vitro. Molds have been designed to allow cell growth in three dimensions (3D). These designs increase cell-cell interactions which assist cells in basic functions\textsuperscript{8}.

1.4 Past Research
In 2014 Justin Clough, a Milwaukee School of Engineering (MSOE) Research Experience for Undergraduates student (REU), designed a mold for 3D cell culture. Each mold contains micro-wells that allow cells to self-assemble into clusters known as “spheroids.” This mold was then tested by REU student Jerusha Kumpati in 2014. Using this design, spheroid formation was recorded after four days\textsuperscript{9}.

1.5 Applications
Because spheroids better replicate liver function, drugs can be administered to the spheroids and any decrease in functionality can be used as an indication of in vivo results. If significant function decrease is found in initial pharmaceutical trials, the drug can be prevented from entering the market. This testing model can also be applied to drugs already on the market. Many drugs have only rare toxic effects; if these are found in the US, the Food and Drug Administration (FDA) can remove it from the market because of potential damage. Drugs affect each person differently because toxicity has many dependant factors. Age, weight, gender and preexisting liver conditions have all been proven as factors effecting liver toxicity\textsuperscript{4}. Obtaining a cell database incorporating these factors could allow for 3D models to test toxicity. Because this model is inexpensive and easily replicable, more factors leading to toxicity could be examined at a faster rate. This would allow for a more individualized prescription process, decreasing the fear of hepatotoxicity as well as hepatotoxicity itself.
2. Methods

2.1 Cell Culture
For all cell cultures, H35 Rat Rueber Hepatoma cells were used. These cells were utilized in previous research and cryopreserved in liquid nitrogen at the Medical College of Wisconsin. Because it has been shown that human hepatocytes do not proliferate in vitro, rat hepatocytes were chosen as they can be easily cultured and are less expensive than human hepatocytes.

2.2 Two-Dimensional Culture
Standard cell culture procedures were followed. Cells were grown in a 1:1 mixture Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 Medium (F12). 10% Fetal Bovine Serum (FBS) and a 1% antibiotics mixture (Penicillin, Streptomycin, L-Glutamine) were added. Cell conditions remained at 37°C and 5% CO₂ for the duration of the culture; cells were passaged every three to four days (or at approximately 90% confluency).

2.3 Three-Dimensional Culture
Wells for the 3D culture were created using a mold designed by Justin Clough. Multiple molds were manufactured at the Rapid Prototyping center (RPC) at MSOE using the Selective Laser Sintering machine (Figure 1. a & b). The molds were placed into a 6-well plate containing a 2% agarose solution and allowed to cure for 45 minutes. Each mold creates 25 micro-wells with a 600 micron diameter and 1100 microns deep (Figure 1.a).

Each gel mold was covered with media and allowed to equalibriate with the media for 24 hours before cell seeding. After this period each well was seeded with 100,000 cells/well. Cells were delivered in 10-20 μL of media containing an additional 10% FBS to increase the density of the solution and assist the cells in settling in the micro-well. Cells were allowed to proliferate for four days (Figure 2) before any drug treatment or testing.

2.4 Neutral Red Dye Test
This initial 2D test was used to determine the optimal concentrations at which hepatocytes experience cytotoxicity. Cells were counted in a Neubauer Chamber and diluted to 4 x 10⁵ cells per mL. Two cell
concentrations were tested: 10,000 cells/well (10K) and 20,000 cells/well (20K). Concentrations of
Acetaminophen (Sigma-Aldrich) were added to each well, ranging from 0.15 mM – 15 mM (therapeutic
centration was estimated to be 6 mM). Plates were incubated and tested at 24 and 48 hours. After
incubation, wells were washed with Phosphate Buffered Saline (PBS) and treated with a Neutral Red Dye
Solution (0.76% dye in media). Cells left living after APAP treatment absorb the Neutral Red Dye. Destain
solution was added to lyse the living cells and release the dye. Absorbance of the remaining solution was
measured at 490 nm. Absorbance is directly proportional to the number of live cells.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)
Functionality was accessed by quantification of albumin secretion, a plasma protein readily produced by the
liver. Albumin concentrations were measured using and Enzyme-linked Immunosorbent Assay (ELISA). Rat
Albumin quantification set was obtained from Bethyl Laboratories. All standards and tests were run according
to manufacturer’s instructions. For initial 2D dye testing the plate was prepared following the concentrations
and treatment periods of the Neutral Red Dye Test. In order to perform 3D testing, APAP concentrations were
added to each of the 6 wells on a standard 6-well plate. Samples were then drawn from each of 25 micro-wells
for testing.

3. Data and Results

3.1 Cytotoxicity
Absorbencies measured at 490 nm were averaged; outliers were removed +/- 1.5 the interquartile range. Data
was also removed in three cases when the absorbance was significantly greater than the highest standard
reading. Data removal was allowed due to the likelihood of human error (plates were pipetted by hand and
contained multiple solutions). Absorbance data was compared to untreated cell samples (with media only) and
summarized in Figure 3.

![Quantitative measurement of cytotoxic effects of Acetaminophen on H35 Hepatoma](image)

Figure 3. Absorbance difference from standard at 490 nm after 24 and 48 hours of
acetaminophen treatment (0.0-15 mM).

At both the 24 and 48 hour treatment periods, the 10K cell concentration showed a greater decrease in
absorbance. The 48 hour treatment period showed larger amounts of cell death at both cell concentrations. At
both 48 hour treatment periods as well as the 10K cell concentration after 24 hours, significant cell death began
at the 4.5 mM APAP concentration.
3.2 Hepatocyte Functional Assay
Absorbencies measured at 450 nm were averaged; outliers were removed +/- 1.5 the interquartile range. Data comparing the absorbancies of 2D at both 24 and 48 hour treatment periods is shown in Figure 4.

Figure 4. Albumin concentration in ng/mL after 24 and 48 hour acetaminophen treatment (0.0-15 mM).

Little to no change in albumin concentration was seen within the 24 hour drug treatment period. Significant albumin decrease was seen after 48 hours within the APAP concentration range of 0.00-2.25 mM. 3D cultures were then treated for 48 hours; results are shown in Figure 5.

Figure 5. Albumin concentration in 2D and 3D cultures after 48 hours of APAP treatment (0.0-15 mM).

Both 2D and 3D cultures showed a decrease in albumin concentration within the 0.00-2.25 mM APAP range. Tests were then repeated and six concentrations of APAP were tested from 0.00-2.25 mM. Because no decrease was shown within 24 hours in 2D cultures, treatment periods were increased to 48 and 72 hours for 3D testing. Albumin concentrations for 3D tests were compared to 2D and are shown in Figure 6.
Conclusions

Cytotoxicity of acetaminophen (APAP) was tested on H35 rat hepatocytes in 2D and 3D in vitro cultures. In 2D cytotoxicity analysis, 10K cells showed a significant decrease at 4.5 mM after 24 hours whereas 20K did not significantly decrease until 15 mM. After 48 hours, both cell concentrations began to show significant decrease at 4.5 mM. The amount of absorbance decrease was larger after 48 hours for both cell concentrations. Initial functionality tests in 2D cultures showed little to no decrease in albumin concentration after only 24 hours of APAP treatment. After 48 hours, albumin decrease could be seen within the 0.00-2.25 mM drug concentration range in both 2D and 3D cultures. After narrowing the drug testing range to 0.00-2.25 mM, a faster rate of albumin decrease was exhibited in the 2D cultures than the 3D cultures. After 48 hours of APAP treatment, 3D cultures showed a significant albumin decrease with only 0.15 mM APAP treatment while the 2D cultures continued to steadily decline within the 0.00-2.25 mM range. After only 0.15 mM treatment, 3D cultures showed an 81.2% decline in albumin concentration. At this same drug concentration, 2D cultures increased the albumin concentration by 0.63%. This suggests that when cells are grown in an environment more similar to that in vivo, they have a higher sensitivity to acetaminophen hepatotoxicity.

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