Effects of Acetylsalicylic Acid on HepG2 Cell Proliferation and Mitochondrial Structure

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Introduction

Acetylsalicylic acid (ASA) is a common treatment for pain, inflammation, and blood clots (Raza and John, 2012). It is also being studied for its potential anticancer effects because it increases cell apoptosis (Jin et al. 2018, Shi et al. 2020). However, it is not completely understood how this increase in cell death is mediated, and it is important to understand the mechanisms better so that the increased apoptosis is restricted to cancerous cells. ASA treatment initiates a cellular stress response, particularly in mitochondria (Raza and John, 2012, Raza et al. 2016). This results in increased reactive oxygen species (ROS) and malfunctions in normal mitochondrial functions like cellular respiration in the cells treated with ASA (Raza and John, 2012, Raza et al. 2016, Uppala et al. 2018). Metabolic changes caused by ASA treatment may also result in mitochondrial membrane structural changes and fragmentation (Uppala et al. 2018). We were interested in studying the effects of ASA treatment on HepG2 cell proliferation and mitochondria, specifically looking at the structure of the mitochondrial membranes. One goal of testing ASA treatment on HepG2 cells was to gain greater insight into how proliferation and mitochondrial function are affected in a variety of cell types. We were also interested in testing higher concentrations of ASA than in these studies to see if this resulted in a more severe stress response and less cell proliferation. Our hypothesis was that treating HepG2 cells with ASA would decrease

their proliferation and that the mitochondria would exhibit visible structural changes, such as membrane fragmentation and aggregation.

Materials and Methods

Cell proliferation assay

Cell proliferation was assessed using the WST-1 assay. 200 µL HepG2 cells from a flask cultured at a 1:6 subculture ratio were grown in wells of a 96-well plate. The set up of the plate, as shown in Figure 1, included 4 columns of control wells (cells treated with DMSO) and 4 columns of wells treated with varying concentrations of ASA. All ASA solutions were made by dissolving the required amount of ASA in DMSO. The cells were incubated in 200 µL of their respective treatment (DMSO or ASA) for 24 hours at 37 °C and 5% CO_2. After incubation, the treatment was removed and 100 µL DMEM media was added. 10 µL WST-1 was added to each well, and the plate was incubated for 30 minutes at room temperature. The absorbance value of each well was measured using a microplate reader in kinetic mode at 450 nm for 60 minutes. After the absorbance values were obtained, the 4 columns of each concentration of treatment were averaged, and the absorbance values for the last time point for both treatment groups were graphed. Non-paired t-tests, ANOVA, and linear regression were used to analyze differences in absorbance values between the ASA and control groups.

Here, our control groups consisted of HepG2 cells treated with DMSO, while our experimental groups consisted of HepG2 cells treated with varying doses of ASA.

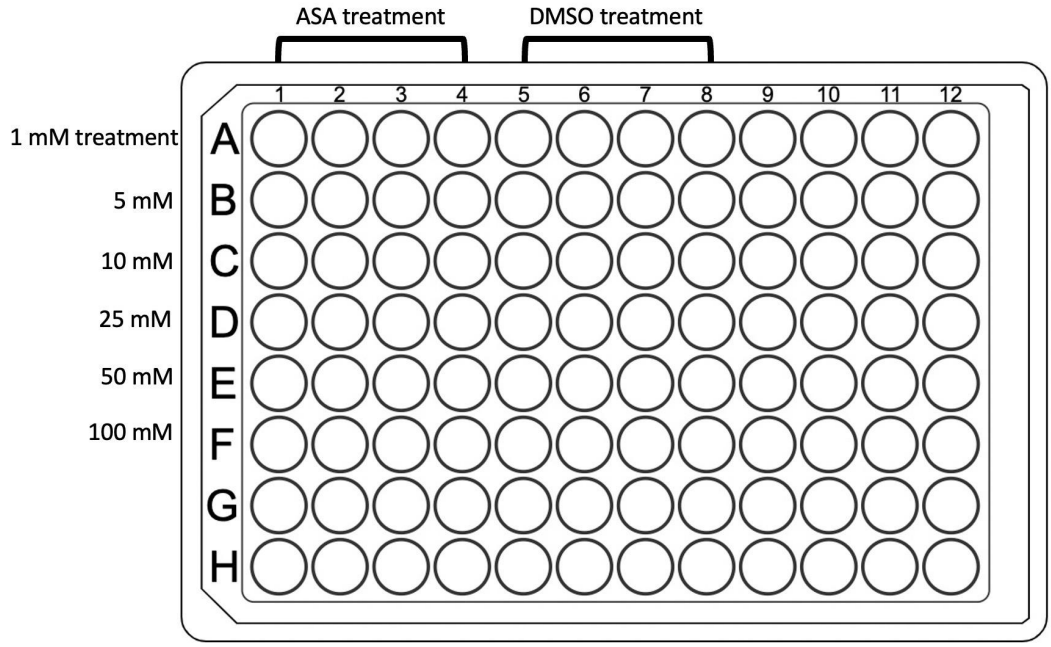

Figure 1. Diagram of 96-well plate setup for cell proliferation assay. 200 µL HepG2 cells were cultured in each well, and the cells were treated with various concentrations of DMSO or ASA.

Cell viability assay

2 mL HepG2 cells were obtained from a flask cultured at a 1:6 subculture ratio. 1 mL cell suspension was treated with 1 mL 100 mM ASA and 1 mL cell suspension was treated with 100 mM DMSO for 24 hours. 0.2 mL was removed from each cell suspension and the cells were stained with 0.2 mL 0.4% trypan blue solution for 5 minutes. Cell viability was assessed using a hemocytometer. 12.5 µL cell suspension was placed in each chamber; the cells in the center and the 4 corner squares were counted. Viable cells stained clear and non-viable cells stained blue. Cell density was calculated using: cells/mL = average number of cells per square x dilution factor x 10^4, and viability was calculated using: viable cells/total cells x 100%.

Here, our control group consisted of HepG2 cells treated with DMSO, while our experimental groups consisted of HepG2 cells treated with 100mM ASA in DMSO.

Fluorescent staining

1 mL (x2) of HepG2 cells obtained from a flask cultured at a 1:6 subculture ratio were grown on circular coverslips in a 3.5 mm dish with an additional 1 mL media. One coverslip of cells was treated with 1 mL 100 mM DMSO and one was treated with 1 mL 100 mM ASA for 24 hours at 37 °C and 5% CO_2. Following treatment, the coverslips were washed 2X with 1 mL PBS; 1 mL fresh PBS was added. A DAPI solution was prepared by adding 2.1 µL 5 mg/mL stock solution to 100 µL PBS, and a mitochondrial orange solution was prepared by adding 10 µL stock solution to 90 µL PBS. 5 µL DAPI and 10 µL mitochondrial orange was added to the coverslips and they were incubated in the dark for 30 minutes. After incubation, the solution was removed and 10 µL PBS was added. The coverslips were transferred to a clean slide, 30 µL media was added, and clean square coverslips were placed over the cell-containing coverslips. The slides were imaged on a fluorescence microscope at 200X and 400X using the UV filter for DAPI and the TX2 filter for mitochondrial orange.

Materials

- HepG2 cell culture
- T25 culture flasks
- DMEM media and trypsin
- Cell culture hood
- Pipet aid
- Pipets

- Acetylsalicylic acid (ASA)
- DMSO
- WST-1 reagent
- DAPI
- Mitochondrial Orange
- Trypan Blue stain
- Micropipettes and pipette tips
- 96-well plates
- Microplate reader
- Fluorescence microscope
- PBS
- Microscope slides and coverslips
- Computer and software to analyze images and create graphs
- Appropriate waste disposal containers

Results

Cell proliferation assay

The absorbance values for the DMSO-treated cells remained fairly constant across all concentrations of treatment. To confirm this, we conducted ANOVA on the absorbance values and found no significant difference ($F = 0.311$, $p = 0.9$) indicating proliferation was stable for the DMSO treatment group. The absorbance values for the ASA-treated cells decreased with increasing treatment concentration, indicating proliferation decreased in a dose dependent manner. Based on paired t-test comparison, the absorbance values for the ASA-treated cells were greater than the

DMSO-treated cells for 1 and 5 mM, approximately the same for 10, 25, and 50 mM, and less for 100 mM, as shown in Figure 2 (respective p-values given in Figure 2). This indicates that the ASA treatment had a beneficial effect on cell proliferation at low doses, did not affect proliferation at moderate doses, and had a negative effect on cell proliferation at the highest dose.

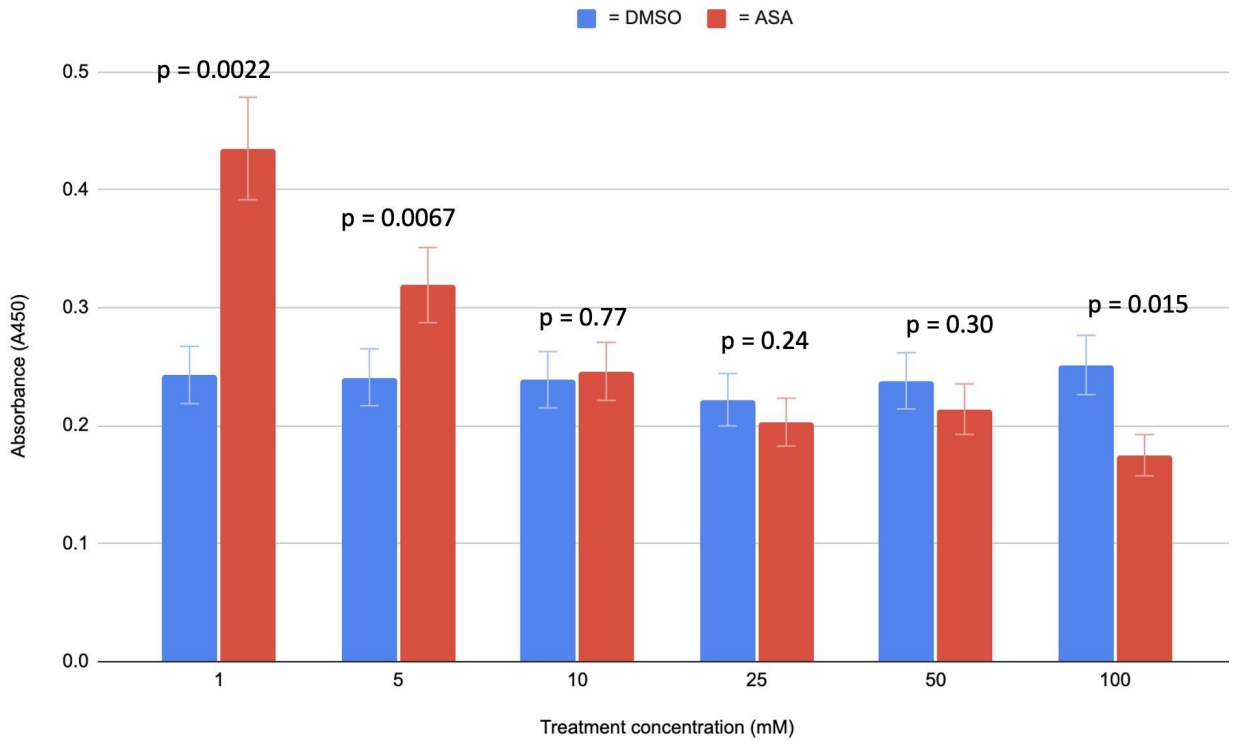

Figure 2. Graph of absorbance values versus concentration of ASA or DMSO treatment. Graph was produced by averaging the absorbance values of the 4 columns of each concentration of treatment at the last time point for both treatment groups. A t-test was performed to determine statistical significance between the DMSO and ASA treatments for all concentrations; the p values are shown.

Additionally, we conducted further analysis on the nature of the decreasing proliferation for ASA-treated cells. Our data follow an exponential decay curve, so we log-transformed the data and conducted a simple linear regression, yielding the equation $y = -0.056 * ln(x) + 0.411$, where y represents the absorbance and x

represents the cell quantity in mM. This linear regression yields an R^2 value of 0.826, indicating a relatively accurate regression.

Cell viability assay

Trypan blue staining was used as an independent measure of cell viability in the ASA and DMSO treated cells. There were fewer cells overall, as well as fewer viable cells, in the ASA treatment group, as shown in Table 1. This verifies our result from the cell proliferation assay at a treatment concentration of 100 mM.

Table 1. Cell density and cell viability values obtained from a trypan blue assay using DMSO versus ASA treated cells.

	Cell density (cells/mL)	Cell viability
DMSO	1.8×10^5	96% viable
ASA	5.8×10^4	52% viable

Fluorescent staining

There were no significant differences in mitochondrial or nuclear structure observed between the ASA and DMSO treatment groups. Representative images are shown in Figure 3. While clear nuclei were shown in DAPI staining, mitochondria did not show clear imaging; we discuss reasons for this in the discussion section.

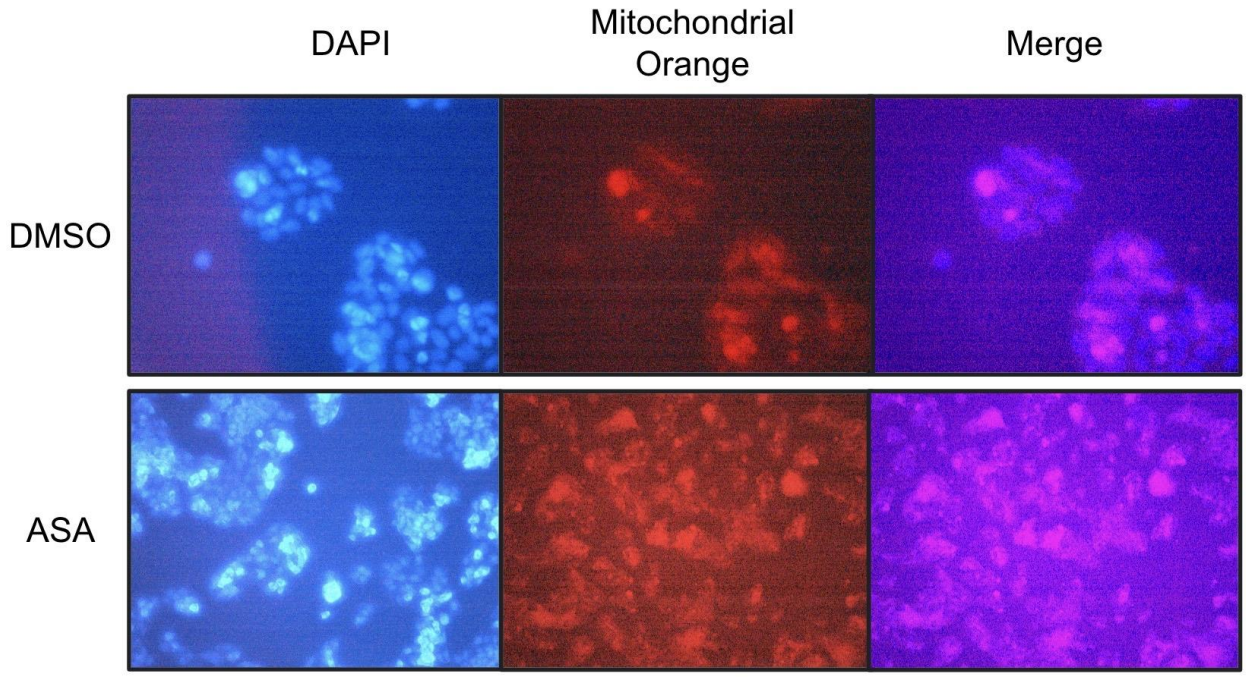

Figure 3. Images of DMSO or ASA treated HepG2 cells stained with DAPI and mitochondrial orange.

Discussion

Our experiments provided further insight into how ASA treatment affects liver cell functions. The ASA-treated cells exhibited a decrease in cell proliferation as the concentration of ASA was increased. The proliferation of the ASA-treated cells was greater than the proliferation of the DMSO-treated cells for 1 and 5 mM, approximately the same for 10, 25, and 50 mM, and less for 100 mM. This did not support our hypothesis that proliferation values would be decreased in ASA-treated cells compared to the control. Possible explanations for this include cell proliferation being promoted by low doses of ASA (Du et al. 2016), error in calculating the correct concentrations of ASA and DMSO, or issues with the ASA being old and having reduced efficacy. The ASA-treated cells displayed significantly less viability than the DMSO-treated cells. The overall number of cells in the ASA treatment group was also less than in the DMSO

treatment group. This supports our results from the cell proliferation assay of the cells treated with 100 mM ASA. The overall decrease in cell number could be due to issues with cell transfer, dead cells getting stuck in the bottom of the tube, or only taking one measurement. We experienced difficulties obtaining clear images using the fluorescence microscope. There appeared to be no difference in the staining intensity or pattern between the treatment groups. This indicates that our hypothesis that the ASA-treated cells would display visible mitochondrial structural changes was not supported, although we would need to obtain clearer images to obtain more conclusive results. Potential causes of the unclear images could be not staining the cells long enough, the stains being old/expired, or the microscope lenses and software not working properly. Overall, we had mixed results from our experiments. Our cell proliferation results appear to somewhat support the data that ASA causes an increase in cell death, however, we saw an increase in proliferation at low doses of ASA. These results indicate that high doses of ASA may be a beneficial treatment option for cancers and diseases resulting from an overproliferation of cells, in addition to its classical uses as a pain reliever. Low doses of ASA might be able to stimulate cell growth and proliferation, which could be potentially useful in regenerative medicine. In future studies, we would like to examine expression of genes involved in mitochondrial function in ASA-treated cells, such as cytochrome c, and see if gene expression levels are altered. This would give us insight into functional changes taking place in the ASA-treated cells in addition to structural changes. It would be interesting to stain for proteasomes in ASA-treated cells, because an additional potential cause of increased apoptosis in these cells is a decrease in proteasome function. This would allow us to

see another type of cellular dysfunction caused by ASA treatment. We also would like to repeat the cell proliferation assay and mitochondrial orange and DAPI staining with different concentrations of ASA and different lengths of treatment.

References

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