Presentation # P1547

Board B559

The Effects of Alcohol on Cell Proliferation and Myotube Formation in Human Female Primary Skeletal Muscle Cells

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Introduction

The impact of alcohol consumption on skeletal muscle tissue in humans has been the subject of considerable study (Garriga, et al., 2000; Nikolic et al., 2012). Previous research has shown numerous harmful effects of pathological alcohol consumption (Bohm et al., 2021), with excess alcohol consumption leading to alcoholic myopathy, which is characterized by muscle weakness and atrophy (Preedy et al., 2001). The primary cause of muscle mass loss is an imbalance in the rate of protein synthesis and breakdown. promoting a greater rate of protein breakdown as opposed to protein synthesis (Steiner and Lang, 2015).

Most of the research that has been done on muscle cells has focused on male muscle cells, animals, or male participants (Beery and Zucker, 2011). One weakness of that earlier research was the absence of investigation into differences in cultures created from male versus female patients (NIH, 2001). Often, these cell lines did not display differences in morphology, but several factors, such as growth rate, time for proliferation and/or differentiation, number of receptors, and total amounts of protein found in cells from males versus cells from females were not considered. However, studies have begun to focus on the difference between male and female cell lines (Gross, et al., 1994, Sibug et al., 1996, Ngwa et al., Alhamyani, A., et al., Bridges et al., 2023).

The main goal of this study was to understand the impact of alcohol use on female skeletal muscle. Specifically, it examined the effect of alcohol on the myocyte proliferation rate and the formation of myotubes in a human female skeletal muscle cell line.(ATCC). Myotube formation is an *in vitro* process which forms multinucleated skeletal muscle fibers through the differentiation and fusion of myoblasts, like the process that occurs *in vivo*. The alcohol levels chosen for this study were based on Garriga et al. (2000), with 0 mM corresponding to sobriety, 10 mM to a social drinker, 25 mM to a heavy drinker, and 100 mM to drinking pathological levels of alcohol.

Our hypothesis is that both cell proliferation and myotube formation will decrease after incubation in low levels of alcohol and severely decrease after incubation in pathological levels of alcohol. To avoid the evaporation of alcohol from cell cultures, the media was changed daily. Experiments were done in triplicate. Cell Proliferation was measured using the CellAssist50 instrument, or by plating cells into T25 cm^2 flasks in the presence or absence of alcohol and then harvesting the cells daily for 5 days. To determine the effect of alcohol on myotube formation, cells were plated onto fibronectin (Sigma Aldrich) coated inserts (Millipore) and incubated in media containing 0-, 10-, 25, -or 100-mM ethanol for 8 to12 days.

Results

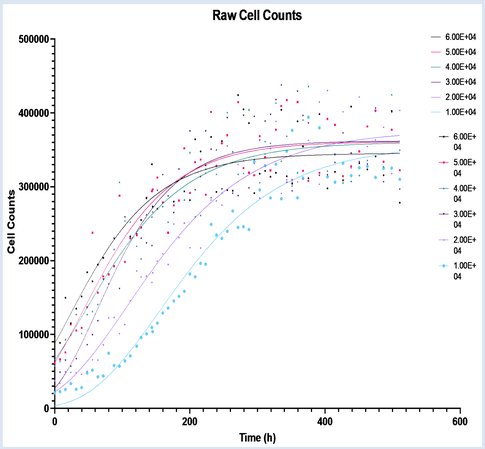

Figure 1. Raw cell count data (scatter points) alongside the Gompertz model fits (solid lines) for human-derived myoblasts seeded at varying initial densities (10,000; 20,000; 30,000; 40,000; 50,000; and 60,000 cells per well). Raw data were collected using automated imaging on the CellAssist50. The model accurately describes growth trajectories, showing reduced lag phases and earlier plateauing for higher densities. Despite these differences, all densities approach a similar maximum population (carrying capacity), highlighting the impact of initial cell density on the timing of growth phases.

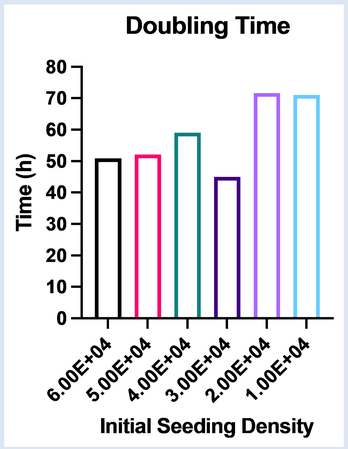

Figure 2. Impact of starting population size on cell proliferation dynamics. The doubling times of human myoblasts seeded at different initial densities, calculated using the Gompertz growth model parameter K, are shown. Higher initial densities exhibited shorter doubling times (e.g., ~45 hours for 60,000 cells/well), indicating faster growth rates, while lower densities showed longer doubling times (e.g., ~70 hours for 10,000 cells/well).

Results

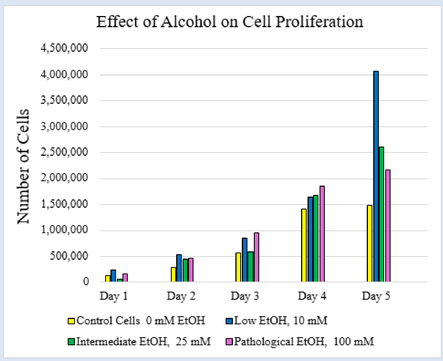

Figure 3. Effect of Alcohol on Cell Proliferation. Cells were grown in complete media containing 0 mM EtOH, 10 mM EtOH, 25 mM EtOH, or 100 mM EtOH for 1 – 5 days. The cell cultures were subsequently trypsinized and the viable number of cells per flask determined using trypan blue and the Bio-Rad TC20 Cell Counter. A one-way ANOVA was conducted which identified a p value of p=0.26, which shows no statistical significance (where significance is p<0.05).

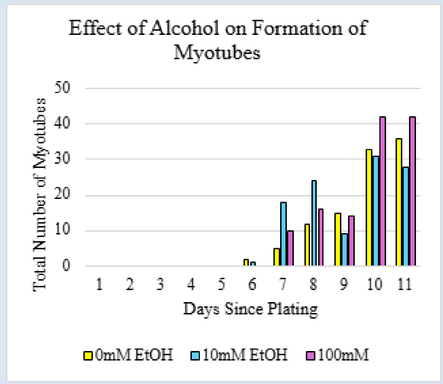

Figure 4. Effect of Alcohol on Formation of Myotubes. To promote the growth of myotubes, 1 x 10^5 cells were plated in complete media onto fibronectin-coated inserts. These inserts were added to a six-well plate divided into three sections. On day 1, the complete media was replaced by differentiation media containing 0 mM EtOH, 10 mM EtOH, or 100 mM EtOH. A two-way ANOVA was conducted which indicated no statistical significance (where significance is p<0.05).

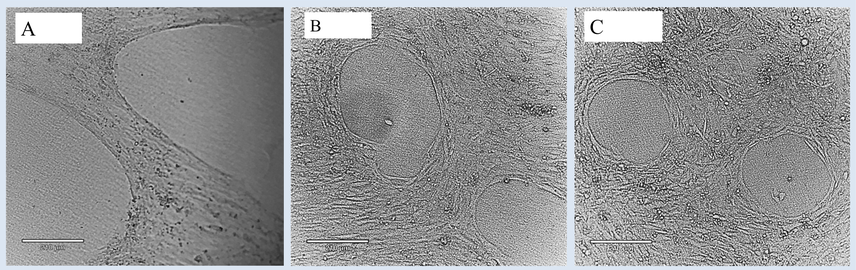

Figure 5. Control and Alcohol treated Myotubes. Cells were grown in differentiation media containing either 0, 10, or 100 mM EtOH for 8 days, A. Myotube incubated with 0 mM EtOH. B. Myotube incubated with 10 mM EtOH. C. Myotube incubated with 100 mM EtOH. Day 5 images taken with the ECHO Revolve Microscope.

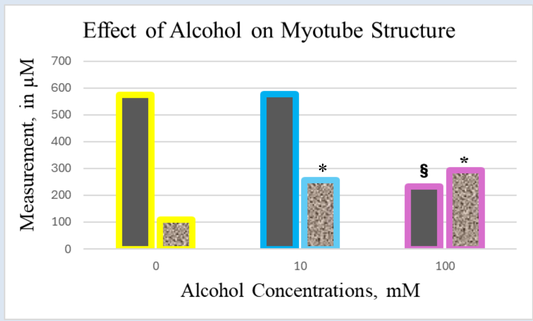

Figure 6. Effect of Alcohol on the Structure of Myotubes. Myotube length and width was measured after 5 days of incubation in differentiation media. Using ANOVA there was a significant difference in length (§) between cells incubated in either 0 mM or 10 mM EtOH and 100 mM EtOH, and in width (*) between 0 mM EtOH, and 10 mM or 100 mM EtOH (where significance is p<0.05). Solid bars = length measurement; Textured bars = width measurement

Results

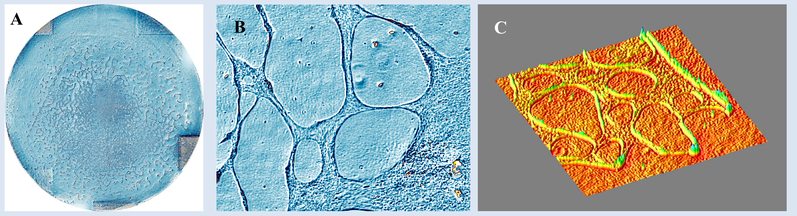

Figure 7. Images of Myoblasts taken using the CellAssist50 Instrument, Myocytes were plated at 1 x 10^6 cells per well onto fibronectin coated inserts in the absence of alcohol. Myotubes began to form 3 days after incubation in differentiation media; images shown are from day 5. A. Full Well QPI Pseudo Color Stitched Image of Myocytes and Myotubes. B. Magnified QPI Pseudo Color Image of Myotubes. C. 3-D rendering of Quantitative Phase Image, showing thickness of myotubes.

Conclusions

The main goal of this study was to understand the impact of exposure to alcohol on female skeletal muscle. Our hypothesis was that both cell proliferation and myotube formation would decrease slightly after incubation in low levels of alcohol and decrease severely after incubation in high levels of alcohol. This hypothesis was partially rejected. The results of our experiments lead to the following conclusions:

Effect of Alcohol on Cell Proliferation:

1. Cells grown in the absence of alcohol exhibited different initial rates of growth, based on initial plating densities. Cells plated at higher densities doubled after ~ 45 hours, while those plated at lower densities doubled after ~70 hours (Figures 1, 2). The effect of plating density diminished over time, with all plating densities achieving confluence.
2. Interestingly, alcohol had no significant effect on cell proliferation. In fact, cells incubated at each level of alcohol seemed to adapt to it, and then used the alcohol as fuel for proliferation, as the number of cells grown in alcohol exceeded the number grown without alcohol (Figure 3).

Effect of Alcohol on Myotube Formation:

1. The presence of high levels of alcohol during myotube formation delayed formation by one day, in preliminary studies (Figure 4). Additionally, low levels of alcohol reduced the number of myotubes formed on day 6, as compared to controls (Figure 4). However, prolonged exposure to alcohol seemed to enhance myotube formation by day 11.
2. There was a structural difference in the myotubes formed in the presence of alcohol, as compared to controls. Myotube length was significantly decreased in myotubes exposed to high levels of alcohol, whereas myotube width was significantly decreased in myotubes exposed to low levels and high levels of alcohol (Figure 6). . Specifically, the length of formed myotubes decreased (control, 573.1 µm vs high alcohol levels, 233.1 µm) while increasing their width (control, 108.48 µm vs. high alcohol levels, 292.75 µm. Figure 5).
3. Using the CellAssist 50 instrument, temporal, high-resolution, whole-well imaging provided comprehensive, real-time visualization of myotube development over the entire surface of the insert. The instrument's automated imaging and data collection capabilities allowed for consistent and repeatable measurements across multiple samples and time points. In addition to QPI pseudo color images, the CellAssist50 can render 3D visualization of myotube thickness (Figure 8).

Future Directions

Image data collected on myotube formation in the presence of control, low, intermediate, and high levels of alcohol over the course of 8 days will be analyzed by a computer algorithm developed specifically to detect myotubes. Cells were imaged every 3 hours for 8 days by the CelllAssist50 instrument. Using the generated algorithm, the number and size dimensions of myotubes formed in the presence or absence of alcohol will be determined, Future experiments will investigate the effect of acute and chronic electronic pulse stimulation on the development of myotubes in this female cell line, grown in the presence or absence of low, intermediate, and high levels of alcohol.

Acknowledgements

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Materials and Methods; References

To access detailed information about the materials and methods used in this study, as well as the references cited, please scan the QR code to the right, for the file labelled, "Windi_ASCB2024-512".