

The Role of Secreted Inflammatory Cytokines (IL-6 and IL-1 β) in Alcohol Addiction

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ABSTRACT

In 2012, 5.1% of the burden of disease and injury worldwide was connected to alcohol consumption, which is why there is reason to believe that it is not just receptor inhibition that results in alcohol's detriments, but rather there are additional ways that it harms the body (specifically the brain). This study aimed to find which cytokines are secreted in the liver as a result of alcohol addiction, and how the cytokines affect the cellular microenvironment, potentially opening the door to many diseases. The hypothesis was two-fold: (1) The IL-6 protein production (inflammation) will increase in alcoholic liver cells (2) The IL-1 β protein (immune response) production will decrease in alcoholic liver cells. The research presented here found that when HepG2 cells were treated with ethanol for 24 hours, cell proliferation decreased by ~120% in the 3% and 4% concentrations, and for 48 hours, cell proliferation decreased by ~110% in the 3% and 4% concentrations. Then, ELISAs were conducted in order to test for IL-6 (* p <0.05 in 3% and 4%) and IL-1 β (* p <0.05 in 3% and 4%) to reveal which particular cytokines are secreted in alcohol-treated cells and at which concentrations they are statistically significant. The ELISAs revealed that IL-6 protein expression increased at 3% and 4% ethanol, while IL-1 β protein expression decreased at 3% and 4% ethanol. The results indicate that alcohol addiction is associated with increased inflammation, which can lead to cell & DNA damage, and decreased immune response, which leads to frequent infection & diseases

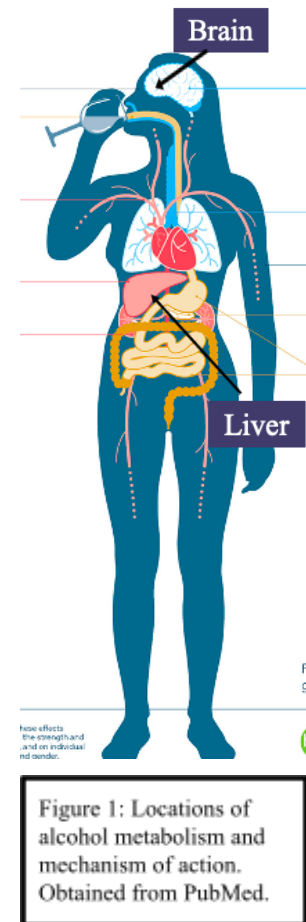
Introduction

Alcohol Addiction

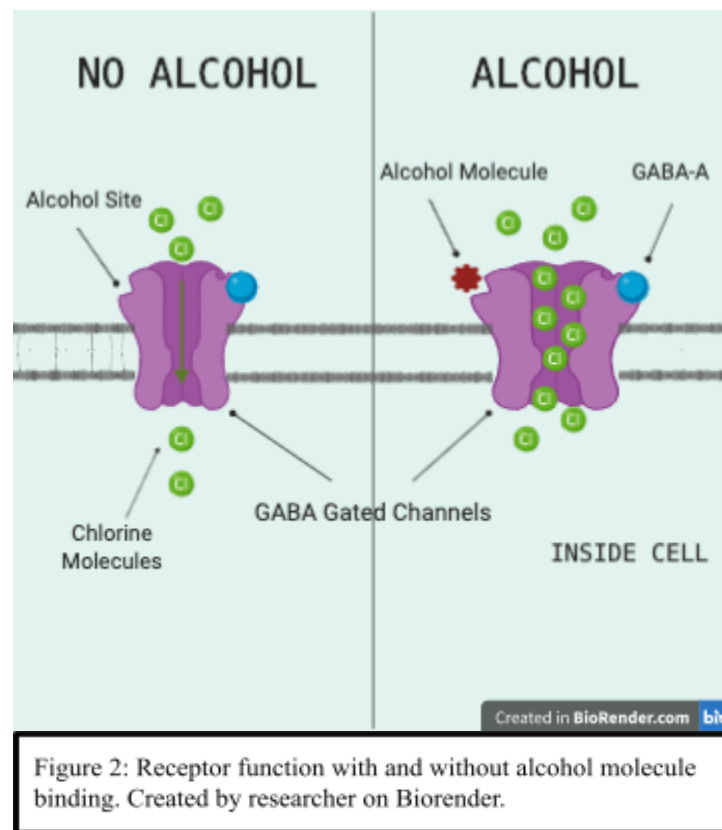
In 2012, 5.1% of the burden of disease and injury worldwide (139 million disability-adjusted life-years) was connected to alcohol consumption. It is the fifth largest risk factor for premature death disability (NIH 2020). Relatively, 15 million Americans suffer from alcohol use disorders, and of those, less than 8 percent receive treatment (Talbot 2020). Alcohol use disorder is defined as a chronic relapsing brain disease characterized by compulsive alcohol use, loss of control over alcohol intake, and a negative emotional state when not using (Alcohol Use Disorders 2020).

Pharmacology

Alcohol interferes with the brain's signaling pathways, specifically the GABA pathway, by mimicking the function of certain neurotransmitters. The GABA A pathway is a metabolic pathway of the inhibitory neurotransmitter GABA and it plays a key role in the development of alcohol use disorders. Alcohol mimics the neurotransmitter's function in the brain, binding to GABA receptors, which are ligand-gated ion channels, and inhibiting neuronal signaling. The GABA receptor has an alcohol site on which the alcohol binds that changes the function of the chlorine ions that pass through the receptor. This change in receptor function results in delusions and inhibits the way the brain works. In the long term, it is seen that when an individual faces withdrawal symptoms, GABA receptors face long term damage and its activity increases in the pleasure center of the brain, making the patient vulnerable to relapse (Scripps 2019). This change in receptor function results in delusions and inhibits the way the brain works. As a result, an addicted individual will experience withdrawal symptoms such as shakiness and dizziness. Not only does alcohol affect the brain, but it also



affects the liver where it is metabolized. Repeated alcohol consumption can lead to liver damage, alcoholic hepatitis, scarring of the liver, and eventually liver cancer.



Cytokines: Cell-to-Cell Communication

Cytokines are defined as signaling proteins that bind to receptors on the outside of a cell and instruct it to perform a certain function. The mechanism of action is as follows: A cell produces a specific type of cytokine in order to signal another cell, the cytokine is released into and travels through the blood, it is received by receptors on the target cell, and the target cell performs a certain function as a result. The target cells can be located all over the body. The process is analogous to sending and receiving a text message. The cytokine-producing liver cell formulates its text message when producing a specific cytokine that relays a specific message. Then, it hits the send button when it releases the cytokine into its extracellular matrix, into the blood. The cytokine travels through the blood to the target organ/cell and binds to a receptor outside the target cell, representing the message being received. Finally, the target cell performs a

specific function based on the message of the cytokine, which is analogous to someone interpreting a received message.

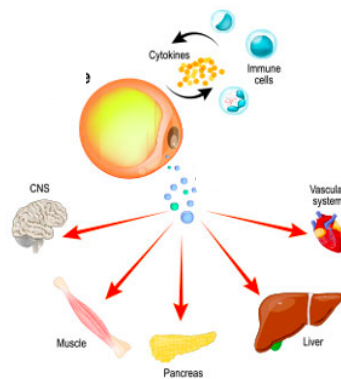


Figure 3: Varying targets of cytokines. Obtained from ScienceDirect.

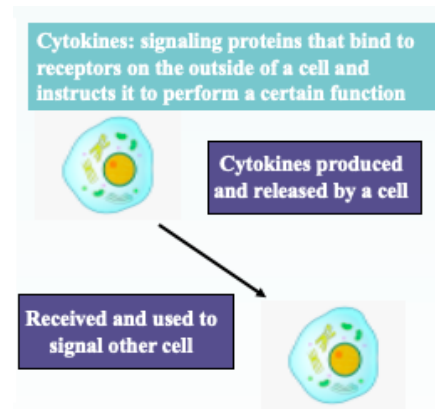


Figure 4: Cytokine mechanism of action. Made by researcher .

Interleukin-6: soluble cytokine with a primary role in signaling inflammation in the body(Tanaka et al. 2014).

(Blood concentration: 0.5 - 5 pg/mL)

Interleukin-1 β : soluble cytokine that signals the lymphocyte to fight infections and signals to leukocytes pass through blood vessel walls to sites of infection (NCI dictionary of Cancer TERMS)

(Blood concentration: 0.5 - 12 pg/mL)

Research Question:

How does alcohol addiction alter the release of cytokines (IL-6 and IL-1 β) and how does the change affect inflammation & immune response?

Hypothesis (Two-fold):

(1)The IL-6 protein production (inflammation) will increase in alcoholic liver cells. (2) The IL-1 β protein (immune response) production will decrease in alcoholic liver cells.

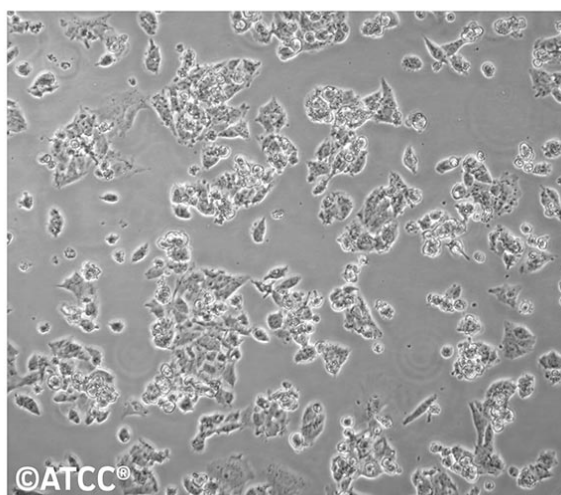
Materials & Methods

Cell Culture:

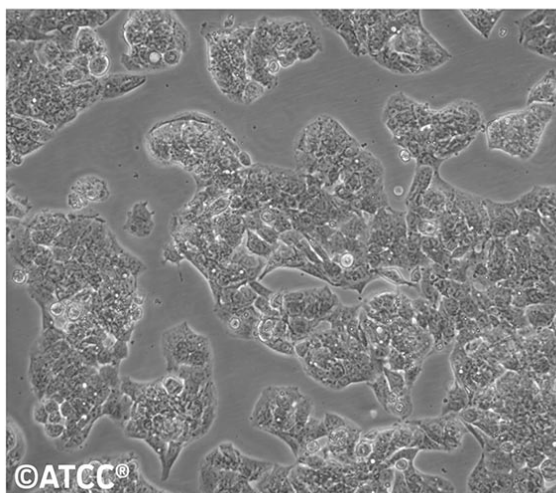
HepG2 human cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle medium and supplemented with 10% fetal bovine serum (ThermoFisher, Waltham, MA).

ATCC Number: **HB-8065**

Designation: **Hep G2**



Low Density



High Density

Figure 5: HepG2 (hepatocellular carcinoma) cells at 100X magnification. Retrieved from ATCC.

Reagents and Treatment:

Ethanol, 200 Proof (100%), 4L (ThermoFisher, Waltham, MA). HepG2 cells will be treated with the following concentrations of ethanol: 1%, 2%, 3%, and 4%. (NOTE: ethanol dissolved in distilled water). The stock solution will be serially diluted in complete culture media to yield each treatment concentration. For example, 10 μ L of 100% ethanol was added to 1 900 μ L of water to yield 1% ethanol. Untreated cells, and cells treated with solvent in complete culture media will be used as negative controls

Trypsinization for Dissociation of Cells:

Trypsin–EDTA solution is used to detach cells from tissue culture dishes and to dissociate cells from one another. Upon confluency, 3 mL of trypsin was added to a T-75 flask of HepG2 and

incubated for 3 minutes at 37°C. If the cells were floating, the trypsin was removed (volume dependent on amount of cells) and neutralized with Dulbecco's modified Eagle medium.

MTS Assay for Measuring Proliferation of Cells:

The MTS tetrazolium compound is bio-reduced by cells into a colored formazan (brown) product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (2).

Assays are performed by adding a small amount of the CellTiter 96® Aqueous One Solution Reagent directly to culture wells, incubating for 1–4 hours and then recording the absorbance at 490 nm with a 96-well plate reader.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA) to Measure Protein Secretion:

Indirect ELISA was used to detect levels of Interleukin 6 and Interleukin 1 beta. After experimentation, plates were spun down at 1000 rpm for 7 minutes. Supernatants were then collected and plated with 100 µl per well for 24 hours at 4°C. Wells were blocked for 10 minutes. The primary antibody for the respective protein was added to each well at 100 µl and allowed to react for 1 hour. 100 µl of HRP-labeled Goat Anti-Rabbit Secondary Antibody was added to each well and allowed to react for 1 hour. 100 µl of ABTS substrate was added and after approximately 30 minutes (when the substrate turned green) the plate was read at 405 nm.

Results & Discussion

Cell Proliferation

The first assay that was completed was the MTS Assay to measure proliferation. This was done to observe what the effect of the concentrations of ethanol would be on the cell survivability. If the concentrations were having little to impact, it would be unlikely that they would reflect results in the ELISAs conducted to detect protein upregulation/downregulation. However, it was even more important to observe if the ethanol was having a negative impact on the survivability. If cells were dying off completely at certain concentrations, the results would be largely skewed,

and the data would not be as prevalent. The concentrations of ethanol used (1% - 4%) were obtained from a literature review that found that human cells would likely only survive well in concentrations up to 4%. In order to avoid large amounts of cell death, only up to 4% ethanol was used. This data is later used to adjust ELISA results, to ensure that survivability does not play a role in comparing the results of secretion of protein. The MTS Assay was conducted over 24 hours and 48 hours to increase data points and accuracy. The results are statistically significant in treatments of 3% and 4%, and indicate a decrease in cell survivability.

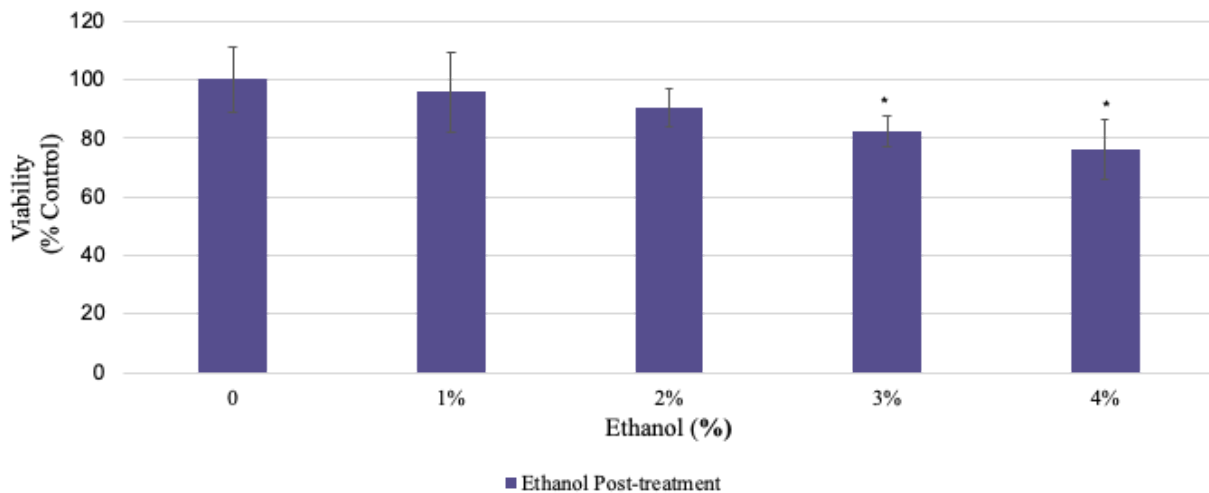


Figure 6: Effect of Alcohol Concentrations on HepG2 Cell Proliferation (24 hours). Cells were treated with 100% ethanol for 24 hours in an effort to measure effects of the treatment on proliferation of liver cells. Data suggests that as the concentration increases, proliferation decreases. Bars are means $\pm$ STDEV (n=5). * = $p < 0.05$ when compared to control.

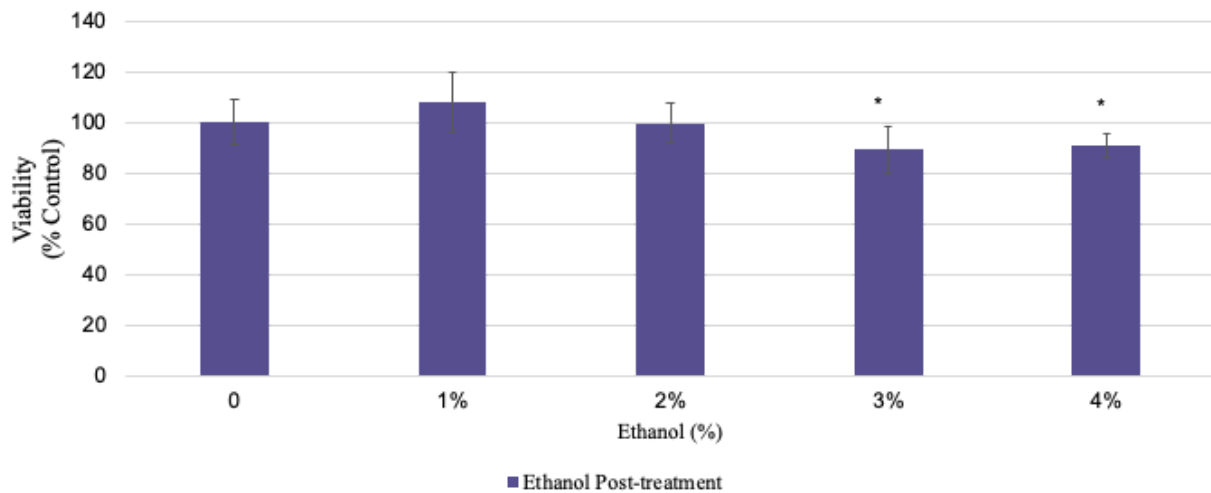


Figure 7: Effect of Alcohol Concentrations on HepG2 Cell Proliferation (48 hours). Cells were treated with 100% ethanol for 48 hours in an effort to measure effects of the treatment on proliferation of liver cells. Data suggests that as the concentration increases, proliferation first increases, then decreases. Bars are means $\pm$ STDEV (n=5). * = $p < 0.05$ when compared to control.

The results of the assay indicate a change in survivability as the concentration of ethanol increased, but the data is steady enough that all concentrations are acceptable concentrations of treatments for the ELISAs and will not gravely affect the results of the protein secretion measurements. The ELISAs will be done over 24 hours, so the results will be based on the survivability of Figure 6, and numbers will be adjusted accordingly.

IL-6 Upregulation

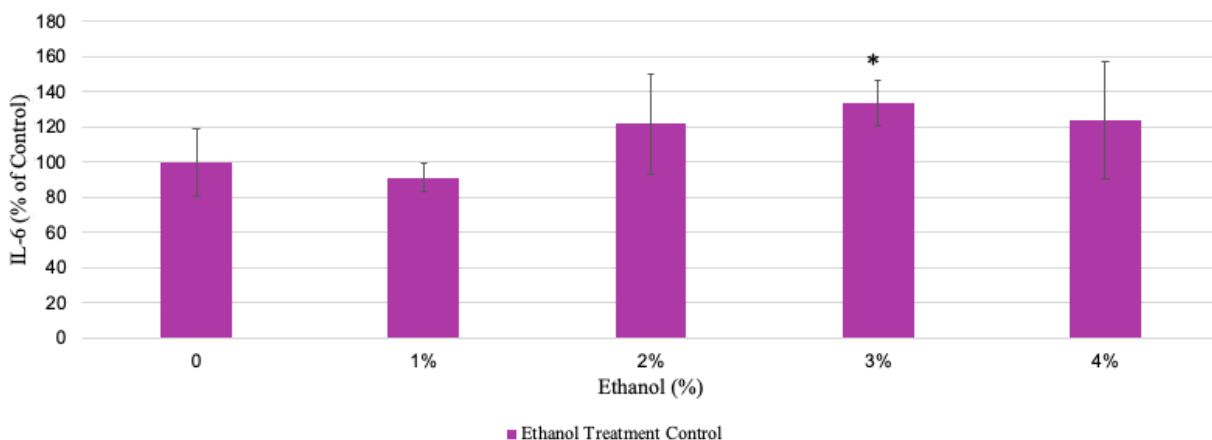
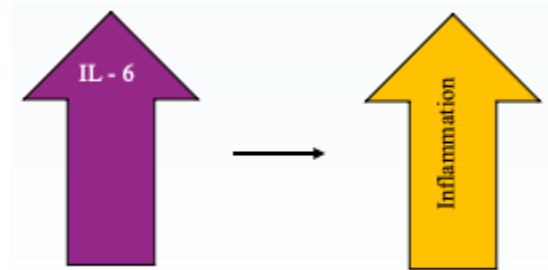


Figure 8: Effect of Alcohol on IL-6 Protein Secretion. Cells were treated with 100% ethanol for 24 hours and then the media was used in an Indirect ELISA to measure IL-6 secretion. Data suggests that as the concentration of ethanol increases, secretion of IL-6 decreases then increases. Bars are means $\pm$ STDEV (n=5). * = $p < 0.05$ when compared to control.

As shown to the right, IL-6 is an inflammatory cytokine, which indicates that an increased concentration of the protein will lead to increased inflammation in the body. As a result, IL-6 upregulation has various indirect consequences for the body. IL-6 is produced in the body, wherever



there is inflammation, either acute or chronic. This includes situations such as trauma, burns, cancers, and infection. This chemical interacts with interleukin-6 receptor alpha, to induce transcription of inflammatory gene products. Additionally, increased IL-6 is associated with the stimulation of acute phase reactions. These promote the activation of innate immunity, keeping tissue damage at bay. When the liver secretes these acute phase reactants, other proteins like albumin and transferrin are necessarily secreted in lesser quantities. (T. Buddy). The IL-6 protein is also associated with obesity and coronary heart disease. Overall, increased inflammation can lead to damage to healthy cells, tissues, and organs, as well as DNA damage and internal scarring. It can also be linked to several diseases, such as cancer.

IL-1 β Downregulation

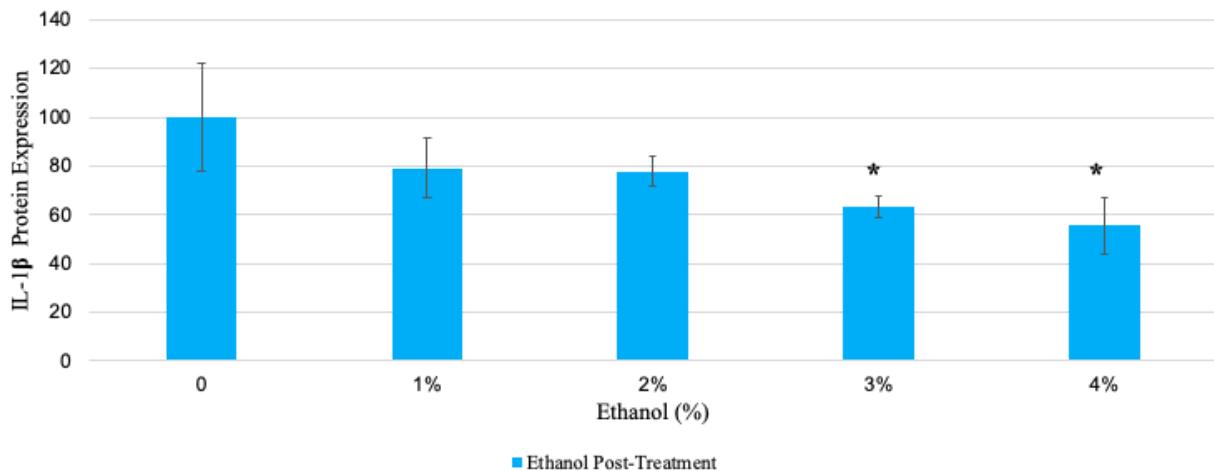
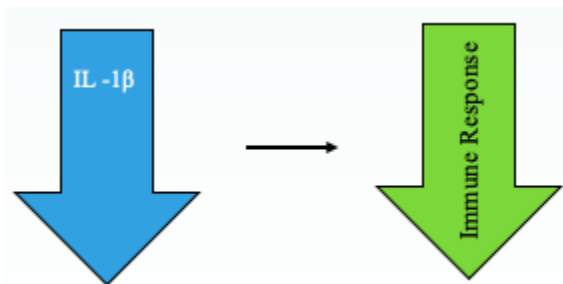


Figure 9: Effect of Alcohol on IL-1 β Protein Secretion. Cells were treated with 100% ethanol for 24 hours and then the media was used in an Indirect ELISA to measure IL-1 β secretion. Data suggests that as the concentration of ethanol increases, secretion of IL-1 β decreases in comparison to the control. Bars are means $\pm$ STDEV (n=5). * = $p < 0.05$ when compared to control.



As shown to the left, IL-1 β is a cytokine that supports immune function by signaling lymphocytes and leukocytes. Specifically, interleukin-1 β is a proinflammatory cytokine that modulates neurotoxic neurotransmission and prolongs kainate-induced seizures by enhancing glutamatergic neurotransmission. Thus, a

downregulation in the IL-1 β protein is associated with decreased immune function and response. Decreased immune response leads to frequent infection, blood disorders, digestive diseases, and organ damage. The IL-1 β protein, itself, has certain positive effects that are decreased when the protein is downregulated. For example, the cytokine aids ovarian function and the inhibition of tumors.

The results reveal that IL-6 protein expression increased at 3% and 4% ethanol and IL-1 β protein expression decreased at 3% and 4% ethanol. These results rely solely on statistical

significance which is obtained by calculating the p-value through a TTEST. These trends are in accordance with the two-fold hypothesis: (1) The IL-6 protein production (inflammation) will increase in alcoholic liver cells. (2) The IL-1 β protein (immune response) production will decrease in alcoholic liver cells. These trends make it evident that biomarkers such as IL-6 & IL-1 β play a key role in perturbing homeostasis in alcoholic liver cells



Figure 10: Direct correlation of alcohol metabolism

Relevant Applications to Biotechnology

Data suggests that upregulation and downregulation of inflammatory cytokines can be indicators of alcohol dependence, as well as immune dysfunction. Thus, in order to track long term and immune health in recovering alcoholics, oral devices can be created. These devices would prick the tongue, collect a small blood sample, and measure blood concentration of IL-6 and IL-1B. If the concentration is concerning low or high, doctors can assess which long term effects an alcoholic patient may face. This device would be crucial in preventative medicine, which is the most effective form of living a disease-free life. Many diseases that occur as a result of upregulation/downregulation, such as cancer, obesity, and blood disorders are difficult to track, but with this new method of understanding potential causes, especially in alcoholic patients, more measures can be taken to mitigate future health problems, that were previously not connected to alcohol.

Future Research

To further understand the effects of alcohol, I would like to experiment with other cytokines such as TNF- α , which is also a cytokine that is produced in liver cells. Additionally, I would also like to work with genes that are associated with alcohol metabolism. Most importantly, genes associated with the GABA pathway, which is the primary neuronal pathway that alcohol affects. Alcohol mimics the neurotransmitter's function in the brain, binding to GABA receptors, which are ligand-gated ion channels, and inhibiting neuronal signaling. The GABA receptor has an alcohol site on which the alcohol binds that changes the function of the chloride ions that pass through the receptor. This change in receptor function results in delusions and inhibits the way the brain works. In the long term, it is seen that when an individual faces withdrawal symptoms, GABA receptors face long term damage and its activity increases in the pleasure center of the brain, making the patient vulnerable to relapse (Scripps 2019).

Conclusion

1. The effect of alcohol is more widespread than just brain signaling and liver cell damage. Societally, it is widely accepted to say "it is just a drink" or "I am not genetically predisposed". However, we all have an immune system and cytokines. Even without predisposition, the risks of alcohol addiction can be severely impactful.
2. Upregulation/downregulation of inflammatory cytokines (IL-6 and IL-1 β), produced in alcoholic liver cells, can lead to a number of different health risks. As mentioned before, we are so used to looking at the macroenvironment, that we often ignore the microenvironment. Micromolecules are opening the doors to a number of different health risks that we may have never associated with alcohol in the first place.

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