

The Effects of Energy Drinks on the Structure and Function of Epithelial Cells and Fibroblasts

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I. Abstract

Energy drinks and their ingredients were studied at the cellular level. This project continued previous research that was conducted at Saint Mary's College of California during the summer of 2010.⁸ Cellular effects of energy drinks were assessed on two cell types: Madin-Darby Canine Kidney (MDCK) cells and rat embryonic fibroblasts. To examine effects of energy drinks on cellular structure and function, cells were treated with Monster Energy and 5-hour Energy. It was discovered that both types of energy drinks negatively impacted cell structure and function in epithelial cells and fibroblasts.

II. Introduction

Energy drink consumption has grown exponentially since the introduction of Red Bull in the United States in 1997. Despite the widespread marketing of energy drinks, the United States does not regulate the ingredients found in energy drinks. The most common ingredients manufacturers include in energy drinks are caffeine, taurine, guaranà, and B vitamins.¹ The FDA defines these ingredients to be "dietary ingredients" so energy drinks are defined to be "dietary supplements" under the Dietary Supplement Health and Education Act of 1994.² This allows manufactures to include supplements in unspecified amounts even if some of the supplements are insufficiently researched.^{2,3}

The lack of regulation of energy drinks has raised concerns. Consumption of energy drinks has been associated with high-risk behaviors such as drug use, alcohol abuse, smoking, fighting, sexual risk taking, and accepting risky dares.¹ Serious adverse effects of energy drinks

have especially been linked to children, adolescents, and young adults diagnosed with conditions like seizures, diabetes, cardiac abnormalities, or behavioral disorders.⁴ Clinical studies show that energy drinks have been associated with cerebral vasculopathy, acute mania, and epilepsy.⁵

The goal of this project was to examine the effects of energy drinks at the cellular level in order to understand the cellular changes underlying the use of energy drink consumption. This study focused on the effects of energy drinks on two types of cells: epithelial cells and fibroblasts. Kidney epithelial cells were chosen for this study because many energy drink ingredients are excreted through urine, which suggests potential deleterious effects on kidney cells. Fibroblasts were chosen because they are present in the connective tissue and are necessary for secreting the extracellular matrix, which helps maintain the structure of tissues. These cells have a dynamic actin cytoskeleton and are known to be important for wound healing.

The results of the project indicate that in both kidney cells and in fibroblasts, the function of the actin cytoskeleton is disrupted without affecting the microtubule cytoskeleton. However, the underlying reason for the disrupted function is different in the two cell types. In the kidney epithelial cells, the actin cytoskeleton is disrupted, whereas the fibroblast showed a normal actin cytoskeleton but aberrant filopodia/lamellopodia formation.

III. Methods

Culturing Cells/ Treatment

MDCK cells and fibroblast cells obtained from ATCC biological resource center were grown in media consisting of MEM containing 10% Fetal Bovine Serum, and 1% penicillin-streptomycin. Cells were maintained at 37°C in an environment of 95% air and 5% CO₂. For imaging, cells were grown on coverslips coated with poly-L-lysine (100 µg/ml). Cells were

treated with Monster Energy and 5-hour Energy. The energy drinks were filtered through a 0.2 micron filter and added to media. An energy drink concentration of 10% was used for the treatment experiments. Since energy drinks are acidic, the energy drink solutions were neutralized by adding drops of 1M NaOH with a transfer pipet. Cells were also treated with caffeine, taurine, and a combination of caffeine and taurine on twelve well plates at concentrations of 40 μ M.

Immunostaining

Cells were treated with energy drinks or their ingredients as described above for a three day period. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% triton and stained with Rhodamine Phalloidine antibody (2.5%) for 2 hours. They were mounted on slides using Fluoromount. The slides were observed using the confocal microscope at UC Berkley.

Wound Healing

Cells were grown on 6 well plates as described above until they reached 100% confluence. The wells were pre-treated with 10% energy drink a day prior to experimentation. A cell scraper was used to make a vertical wound from top to bottom of each well. The wounds were observed in a 1 cm² area near the center of the well. Throughout the duration of the wound healing process, the wells were photographed. A calibration slide was photographed and Image J (NIH) software was used to measure its wound distance in micrometers. Using the calibrated software, four wound distance measurements were taken and averaged for each photograph.

Western Blot

Cells were grown in large flasks until they reached 100% confluence. Control flasks were fed with media and experimental flasks were treated with 10% 5-hour Energy. After a three day treatment, the proteins of the cells were extracted by lysing the cells with buffer containing water, 1% SDS, 5% TRIS-HCL, 2% PMSF, 2% 2-mercaptoethanol. The buffer solution was scraped throughout the flask and then transferred with a syringe to a centrifuge tubes. The tubes were boiled for three minutes and then centrifuged for 15 minutes. To determine concentrations, a protein assay was performed. 65µl of sample was mixed with sample buffer and run on a 4-12% Bis-Tris gel at 200 V for 35 min. The proteins were transferred to a nitrocellulose membrane. The proteins were detected by standard immunoblotting procedure (NuPAGE Technical Guide provided by Invitrogen).

The membrane was rinsed, blocked with 5% non fat milk for 1 hour, followed by incubation with an anti-actin antibody (.5%). The staining was detected by incubating with a streptavidin conjugated secondary antibody (1%) and visualized by staining with diaminobenzidine.

IV. Results

MDCK Cells Show a disruption in the Actin Cytoskeleton Structure

In cells, actin cytoskeleton networks appear as cables running across cells. In this experiment, the actin of the MDCK cells was stained with rhodamine phalloidin in order to see how energy drinks impact the actin cables within cells. Cells treated with control media showed a clearly defined actin cytoskeleton. When treated with Monster Energy and 5-hour Energy, cells displayed actin cytoskeleton problems. Figure 1 compares the number of “normal” and “disrupted” cells among control, Monster Energy, and 5-hour Energy.

The control MDCK cells in Figure 2A show distinguished cells containing clear actin cables. Figure 2B shows MDCK cells treated with 10% Monster Energy. Compared to control, Monster Energy treated cells do not have visible actin cytoskeleton networks. It was observed that larger MDCK cells in Monster Energy treated fields displayed a visibly normal actin cytoskeleton while the majority of cells, the smaller MDCK cells, experienced a significant structural disruption. Cells treated with 5-hour Energy displayed less actin cables compared to control (Figure 2C). Compared to control, the actin cables of the 5-hour Energy treated cells are elongated and have a more web-like structure. Stack images of the 5-hour Energy MDCK cells reveal that the actin cables extend past the plasmamembranes of the cells (Appendix A). A stack photograph displays images of the same viewing field at different depth layers.

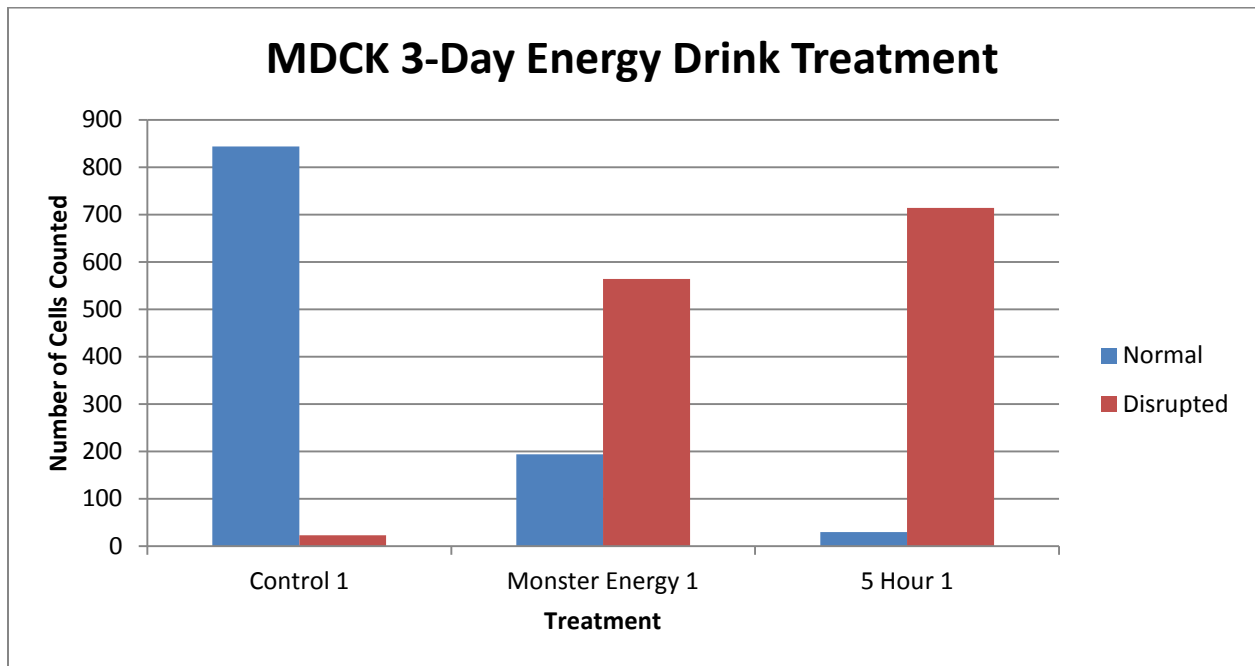


Figure 1

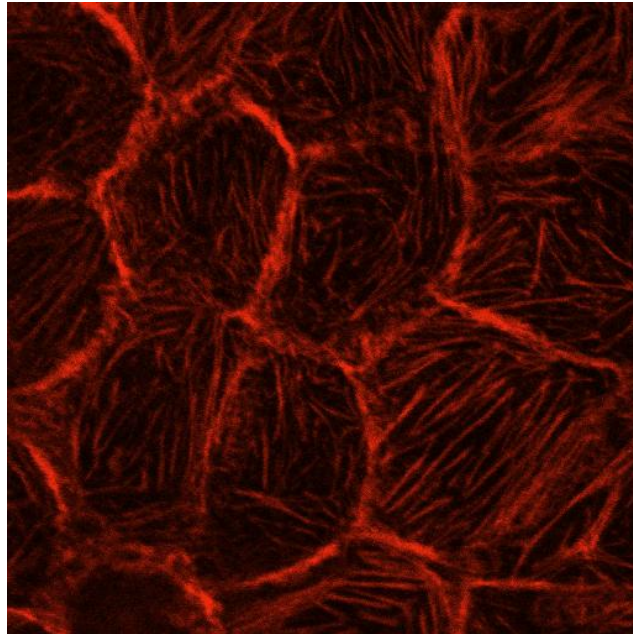


Figure 2A: Control

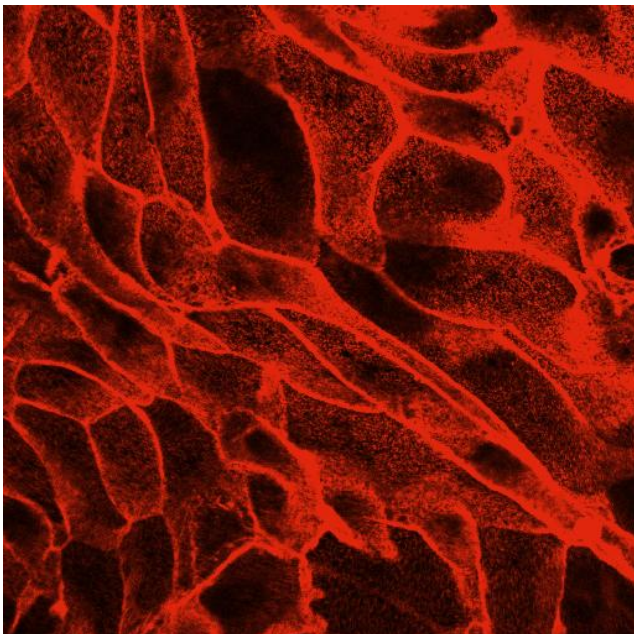


Figure 2B: Monster Energy

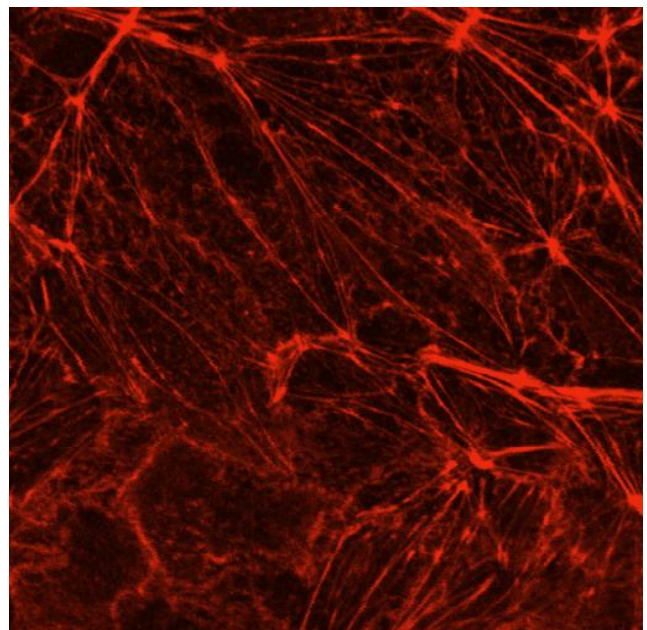


Figure 1C: 5-hour Energy

Figure 2: MDCK 3-Day Treatment

MDCK Cells Experience a Delay in Wound Healing

After observing a disruption in the actin cytoskeleton of MDCK cells, experiments were designed to test cellular function, specifically functions of the actin cytoskeleton. A wound healing experiment was designed to test whether disrupted actin cytoskeleton networks would impact cell function. A five day wound healing experiment of 10% energy drinks on MDCK cells caused the cells to have a delay in their wound healing process. Figure 3 below shows that the wounded control cells are almost entirely healed within 19.25 hours after the cells were scraped. The control cells were fully confluent by the third day of the experiment. The wound distance of the MDCK cells treated with Monster Energy cells was observed to be healing, but a slower rate compared to control. This is illustrated by the less steep slope of Monster Energy in Figure 3. The data for 5-hour Energy shows that the wound distance increased at 19.25 hours for Wound 2 Experiment. However, the wound distance did not actually increase. The increase in measurement was caused by the slightly different reference points of the photographs. It can be understood from the 5-hour Energy data that there was no apparent function in wound healing.

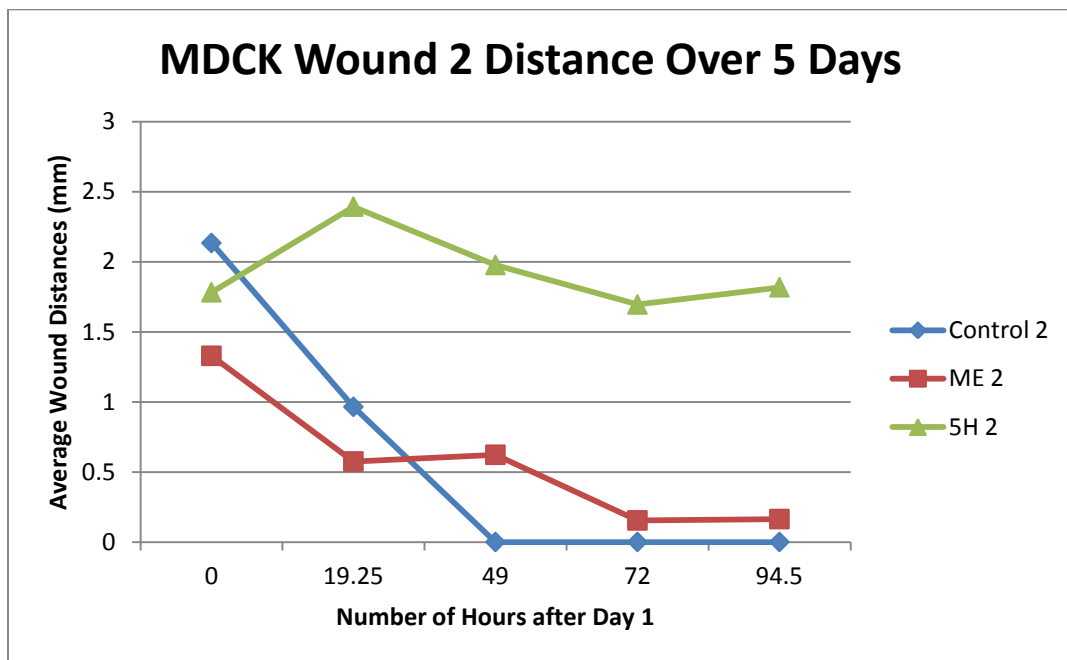


Figure 3

Fibroblasts Experience a Delay in Wound Healing

Fibroblasts treated with 10% energy drink concentrations did not experience disruptions in the actin cytoskeleton network. A wound healing experiment was carried out for fibroblasts in order to assess if cell function would correlate with the observations of normal actin cytoskeletons in treated fibroblasts.

Fibroblasts that were treated with 10% energy drink experienced a delay in their wound healing process. Over the 4 day period of the experiment, only the control cells fully healed. Figure 4 shows that the control cells are almost entirely confluent 19 hours after the scrape, while Monster Energy and 5-hour Energy treated cells did not fully heal after 67 hours. The slopes displayed in the graphed data of Figure 4 show that 5-hour energy delayed wound healing function more than Monster Energy delayed wound healing function. The pictures in Appendix B of the fibroblasts show qualitatively that Monster Energy and 5-Hour healed slower than control.

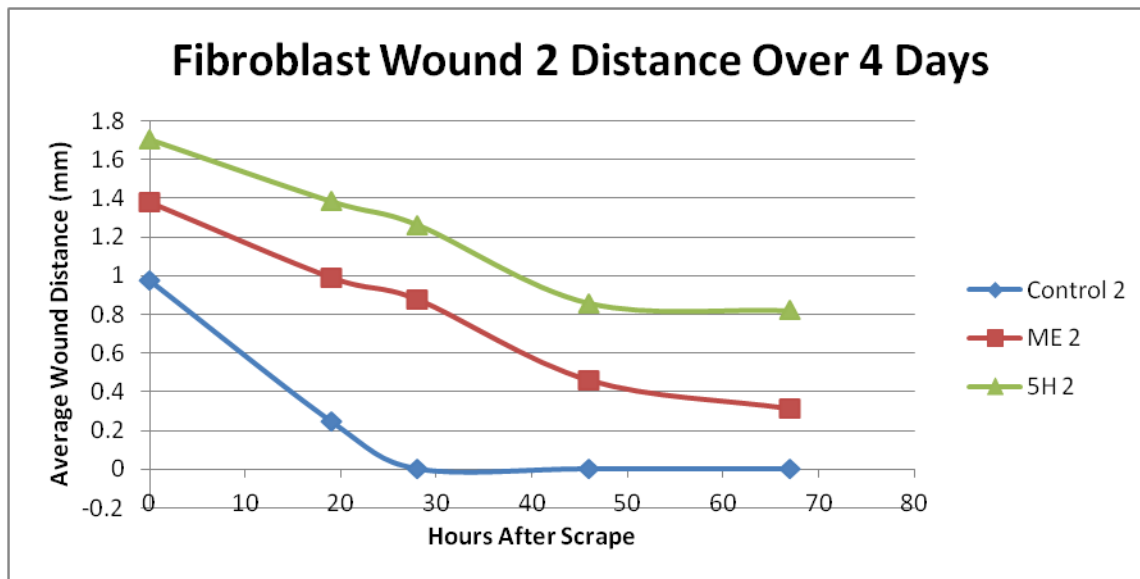


Figure 4

Fibroblasts Display a Change in Membrane Dynamics

In an attempt to find a mechanism for the delayed wound healing process, the wound edges of fibroblasts were more closely observed. Upon closer examination at higher magnification (40x), the fibroblasts showed significant differences in membrane dynamics. Figure 5A shows the edge of the control cells, which display a normal ruffling of edges. These ruffled edges (darker areas of edge) are caused by filopodia/lamellopodia extensions that the cells use to crawl. Figure 5B shows the smooth edges of Monster Energy treated cells. These cells responded to the Monster Energy treatment by not putting out filopodia/lamellopodia extensions at the edge of the wound. Figure 5C shows aberrant formation of filopodia/lamellopodia. The 5-hour Energy treated cells show thin, over extensions of the membrane along the edge of the wound.

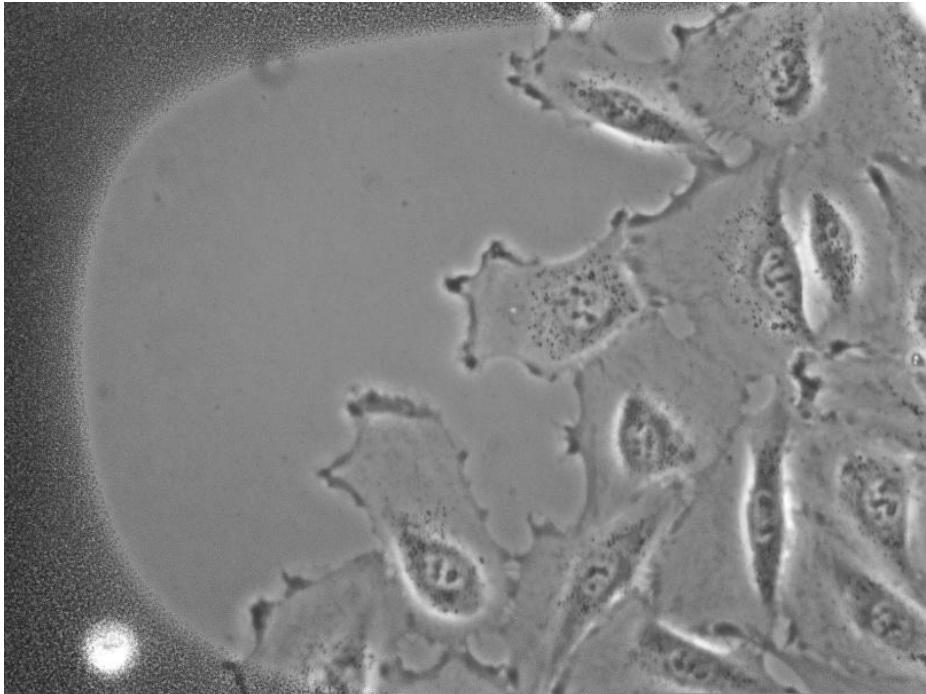


Figure 5A: Control (40x)

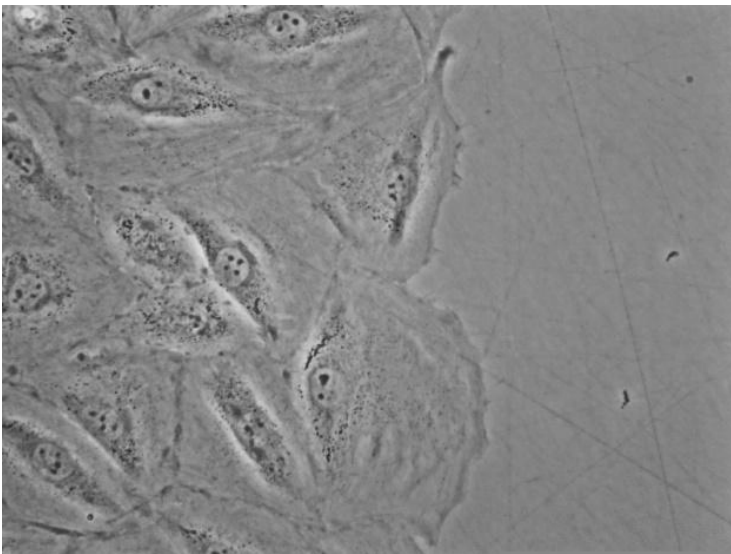


Figure 5B: Monster Energy (40x)

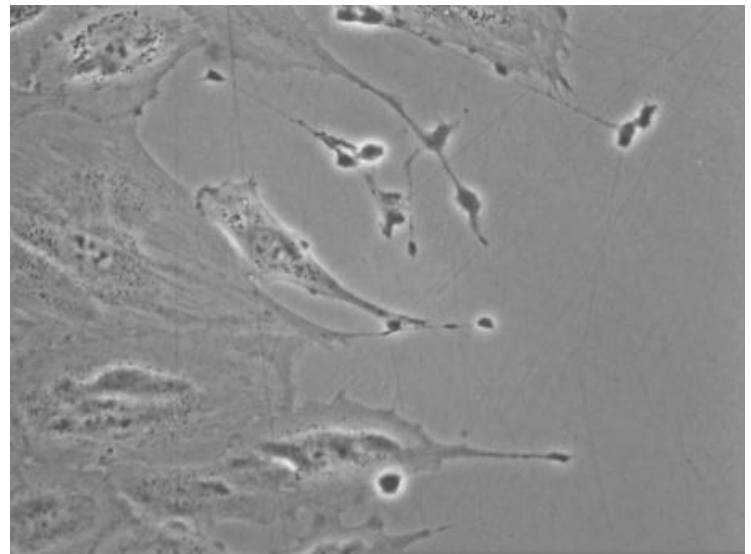


Figure 5C: 5-hour Energy (40x)

Western Blot Shows Unchanged Actin levels in Fibroblasts

A western blot experiment was performed to assess the actin levels in the fibroblasts. The western blot protein samples were taken from control fibroblast proteins and 5-hour Energy treated fibroblasts. After staining the actin, the nitrocellulose membrane showed a band of actin from control and a band of actin from 5-hour Energy (Figure 6). The ladder shown on the right side of the membrane confirmed that the two bands were actin bands located at about 60kDa (kilodaltons). Compared to the control band, the 5-hour Energy displayed a normal actin level.

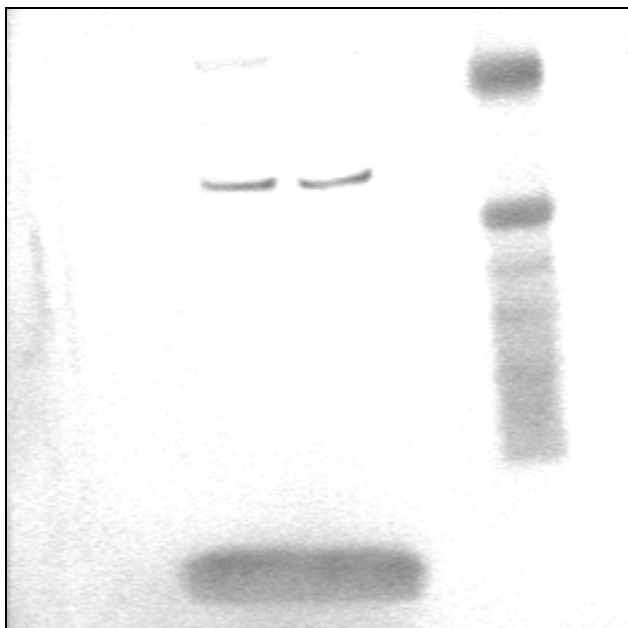


Figure 6: Nitrocellulose membrane depicting a ladder, and actin bands from Control and 5-hour Energy

V. Discussion

The widespread use of energy drinks can be seen on a daily basis. For example, energy drink consumption is especially popular among college students. Reasons for student consumption include increasing alertness for morning classes, boosting athletic performance, and pulling “all-nighters.” For how much these unregulated beverages are being consumed, there has been insufficient research done at the cellular level. Some of energy drinks more common ingredients, such as caffeine and taurine, have been individually studied. Excess caffeine consumption leads to caffeine intoxication, which has symptoms of nervousness, anxiety, restlessness, insomnia, gastrointestinal upset, tremors, tachycardia, and psychomotor agitation.³ Taurine is involved in multiple metabolic processes such as, osmoregulation, antioxidation, and glycolysis.^{6,7} Taurine is used as a supplement because it is thought to promote biliary health, eye health, and prevent congestive heart failure.⁵

Even though some ingredients in energy drinks have been studied individually, the ingestion of ingredients in high quantities and in combination has yet to be adequately researched. In this project, cells were treated caffeine and taurine at 40 μ M, individually and in combination. Unlike energy drink treatments, caffeine and taurine produced no noticeable cytoskeleton disruptions in MDCK cells or a delay in wound healing in fibroblasts. These data show that effect of energy drinks on structure and function on the two cell lines must be attributed to other ingredients, or an excess of caffeine and taurine in combination with other ingredients. As long as consumers desire the energy source from these beverages, energy drink companies will continue to provide these beverages containing unregulated ingredients in unregulated amounts.

Epithelial Cells

Treating MDCK cells with energy drinks showed disruptions of the actin cytoskeleton networks in cells. While energy drinks may provide a consumer with desired energy, they could possibly be disrupting kidney epithelial tissue. If epithelial tissue is exposed to a high enough concentration of energy drink ingredients, the data from this project suggests that the actin cytoskeleton structure of the cells would be disrupted. The data above shows that 5-hour Energy more significantly disrupted MDCK actin cytoskeleton structure. Upon further examination with the confocal microscope, it was discovered that the cells that Monster Energy treated cells that were disrupted were much smaller than the Monster Energy treated cells with normal actin cytoskeletons. After referring to the ATCC website, it was understood that the MDCK cell line had two types of cells: tube and dome.

The data show that Monster Energy affected the tube cells more severely than the larger dome cells. Extrapolating this data, Monster Energy might affect a certain part of the kidney epithelia more severely than another part. Compared to Monster Energy, 5-hour Energy disrupted the cells more uniformly and had a more severe affect on the wound healing process. The difference in severity in cell disruption and cell function could be possibly due to a dose response between the two drinks. A 1.93 ounce bottle of 5-hour Energy relatively produces the same desired energetic effects for a consumer as a 16 ounce can of Monster Energy. The difference in severity between the two energy drinks on cell sheets may point to the results of more concentrated energy drink ingredients in the human body from excessive consumption or long term consumption.

In addition to cell structure, actin filaments are essential for dynamic cellular processes such as cell movement, phagocytosis, and cell division. Wound healing experiments were

designed for MDCK cells to assess whether a disrupted structure would influence a dynamic cellular process such as wound healing. The wound healing process requires functional actin cytoskeleton networks in cells. A sheet of cells heals by crawling across a wounded surface and by dividing until the surface is fully confluent. This crawling depends on lamellipodia and filopodia, which are actin extensions on the leading edge of the cell that drag the cell across the wound. It was discovered that energy drinks inhibit the wound healing process in MDCK cells.

Both of the popularly consumed Monster Energy and 5-hour Energy drinks caused a delay in wound healing. The purpose of this experiment was not to inform consumers about whether they should treat a wound with an energy drink; instead it was to analyze the effects energy drinks have on cellular function. It was discovered that energy drinks cause a delay in wound healing function for epithelial cells. Just as kidney epithelial tissue that is exposed to significant concentrations of energy drinks can have an effect on actin structure, it can also have an effect on actin related processes.

Fibroblasts

Like epithelial cells, energy drink treatments on fibroblasts caused a delay in wound healing function. However, it was discovered that fibroblasts did not experience a delay because of a disrupted actin cytoskeleton; instead, the data suggests a change in membrane dynamics caused the delay. This shows the delay energy drinks cause in wound healing does not exclusively correlate with a disruption in actin cytoskeleton structure. Extrapolating these results, energy drinks could possibly inhibit actin dependent cellular processes without displaying a significant actin disruption in the body.

Studies have shown that proteins such as CDC-42, Rac, and Rho have an active role in actin polymerization and depolymerization.¹⁰ The difference in membrane dynamics between control, Monster Energy, and 5-hour Energy could be possibly caused by the energy drinks disrupting actin-binding proteins. The data from the Western Blot experiment show that the actin levels of the treated fibroblast cells were unchanged. The experiment showed that a lack of actin was not causing a change in membrane dynamics, which further suggests that the disruption of actin-binding proteins are the cause of the change in membrane dynamics. Consuming energy drinks may not reduce actin levels in fibroblasts, but could have an affect on actin-binding proteins necessary for actin related processes in the human body.

VI. Conclusion

Monster Energy and 5-hour Energy cause a disruption in epithelial actin cytoskeletons and a change in fibroblast membrane dynamics. Both epithelial cells and fibroblasts experience a delay in wound healing when treated with the two energy drinks. 5-hour Energy treatments had a more negative effect on cell structure and function than Monster Energy for both cell types. While energy drinks negatively impacted structure and function of cells, the ingredients caffeine and taurine did not.

VII. Acknowledgements

Dr. Vidya Chandrasekaran for all her guidance and support throughout the summer and school year. Charlotte Lea for taking care of the MDCK cells for two weeks and for repeating a fibroblast wound healing experiment. Amy Bockman for ordering the antibodies and kits necessary for the project. The Science Department of Saint Mary's college of California for this opportunity to do undergraduate research.

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Appendix A: 5-hour Energy Stack Image

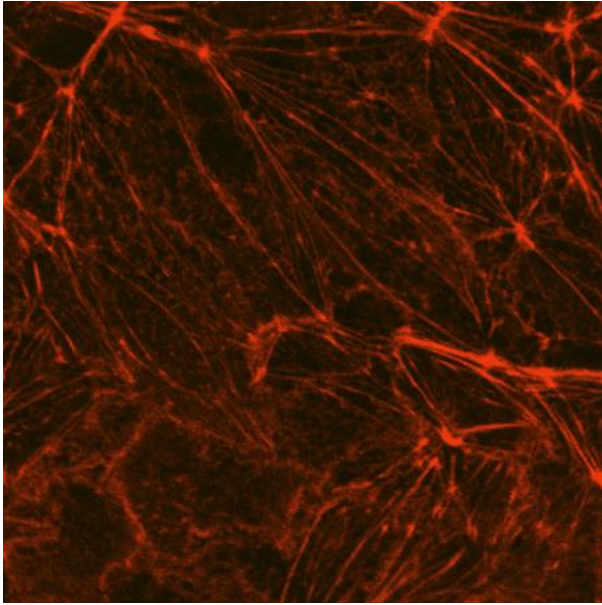


Figure A: 5-hour Energy at 2nm

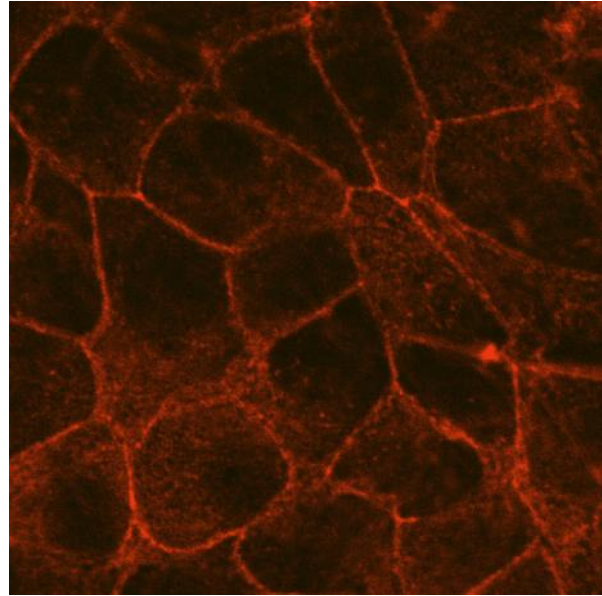


Figure B: 5-hour Energy at 5nm

Appendix B: Fibroblast Wound Healing (4x)

Control

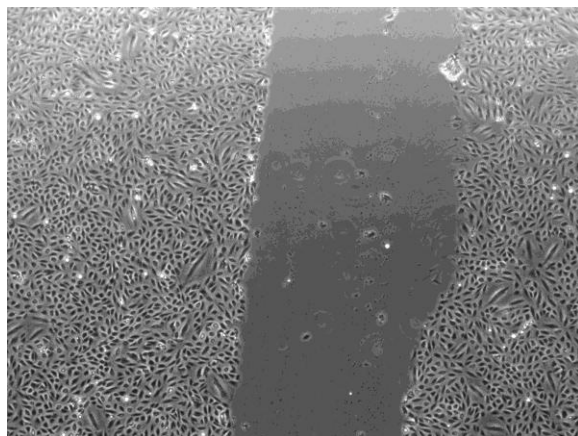
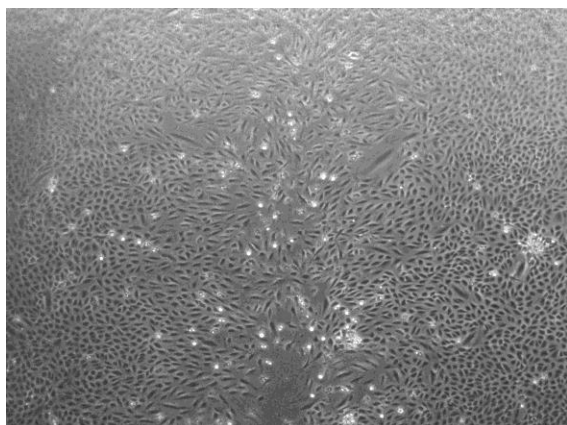
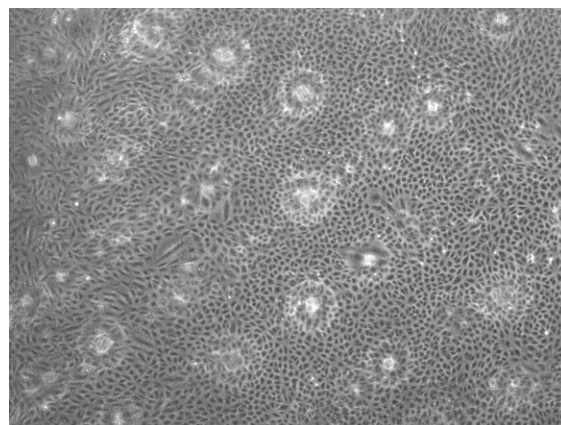


Figure A: Initial Scrape



19 Hours After Scrape



28 Hours After Scrape

Monster Energy

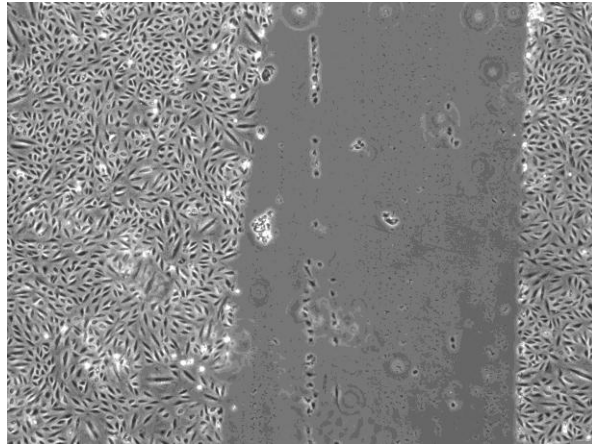


Figure A: Initial Scrape

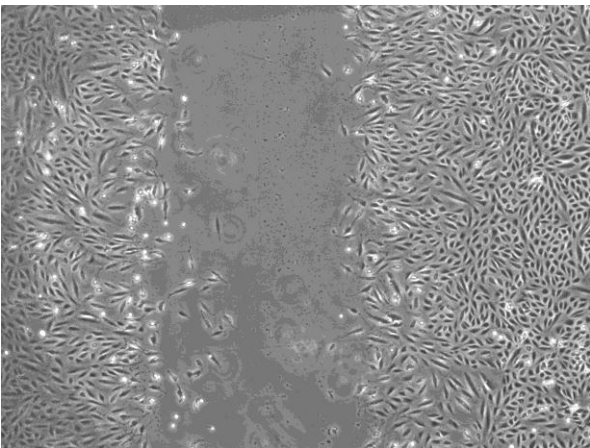


Figure B: 19 Hours after Scrape

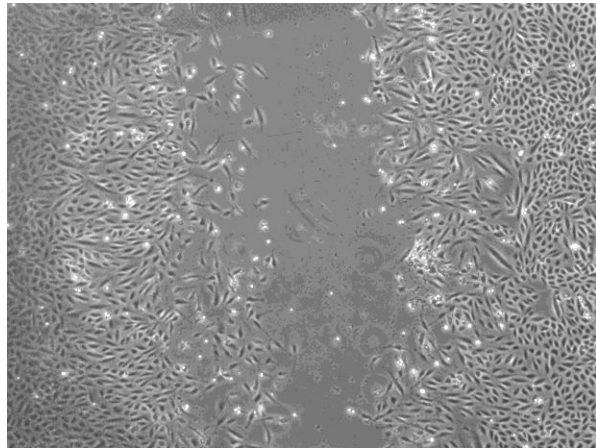


Figure C: 28 Hours after Scrape

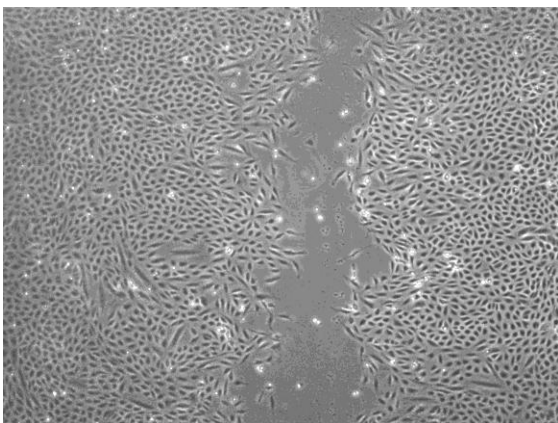


Figure D: 46 Hours after Scrape

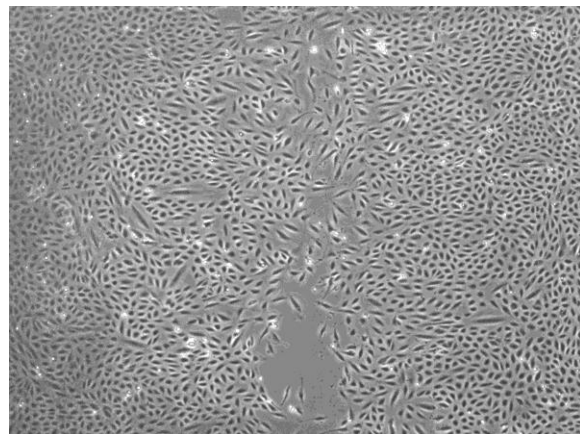


Figure E: 67 Hours after Scrape

5-hour Energy

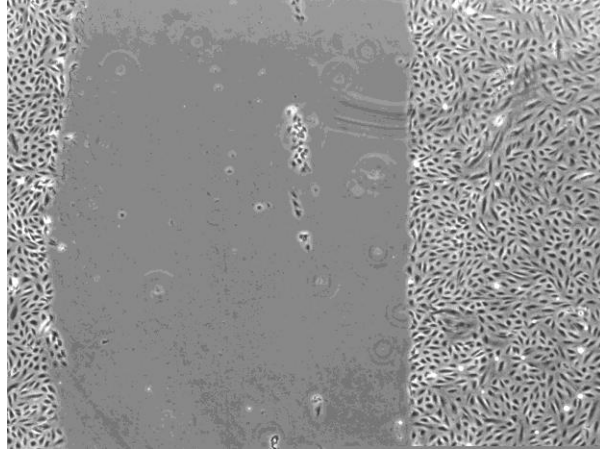


Figure A: Initial Scrape

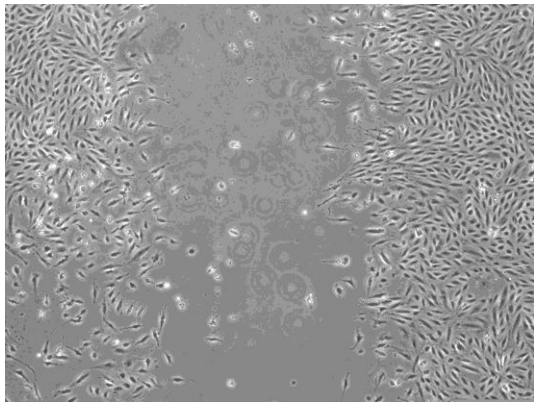


Figure B: 19 Hours after Scrape

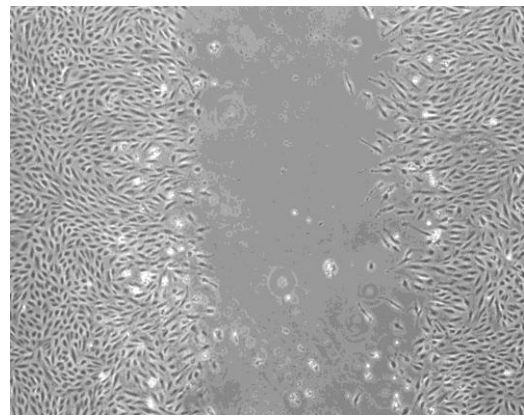


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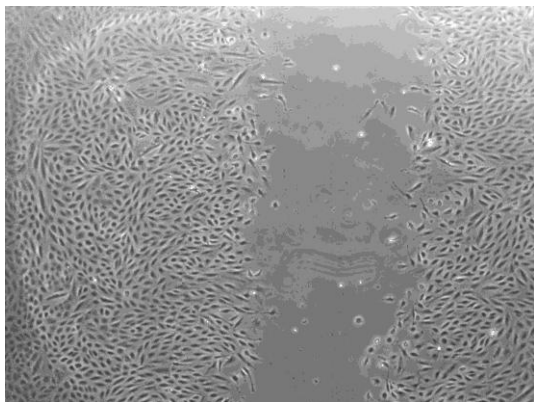


Figure D: 46 Hours after Scrape

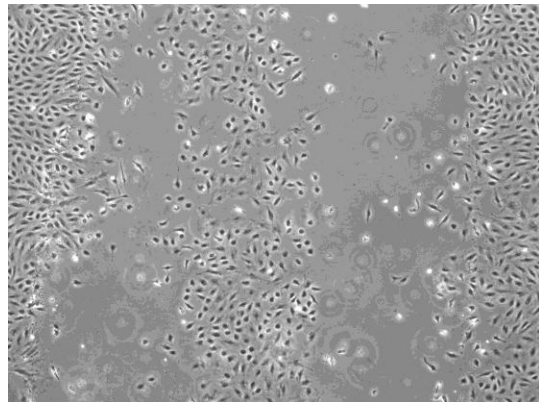


Figure E: 67 Hours after Scrape