TEM Analysis of Caffeine-Treated HT-29 Cells to Investigate Metabolic Stress

Mollee Brummond
R.Y.
E.R.
S.M.
S.P.

Background

This semester, MRE'SS has been working with HT-29 colon cancer cells and their response to caffeine treatments to determine the drug's toxicity. Colorectal cancer has become increasingly prevalent in people younger than 50 with 20% of colorectal cancer diagnoses in 2023 belonging to people under the age of 55 [1]. The concept of caffeine as an anticancer agent [2] has become a promising potential form of therapy in the field of cancer treatment/therapy. Coffee, a beverage that is naturally high in caffeine, has been shown to reduce odds of developing colorectal cancer by 26%. Relative to current cancer therapy drugs, coffee is a more cost-effective drug and easily sourced, which would help with increased accessibility [3]. Research carried out by Chen & Hwang and Liu et al. used concentrations up to 8 mM to view caffeine effects on their cells [4, 5]. In Chen & Hwang's study, 2 mM concentration caused a significant decrease in cell survival, with 2 mM having a 10.3% survival while 1 mM was at 54.5% survival and 0.5 at 67.3%. Liu et al.'s study showed that 2 mM had less than 60% cell viability compared to 1 mM and 0.5 mM, which had viabilities of less than 75% and 90%, respectively.

There is also a widespread prevalence of caffeine usage in college students and working adults that makes understanding its cytotoxic effects significant to public health. Caffeine holds a significant role in many cultural spheres, most prominently in school and work culture. The prevalence of cafes in the United States not only has a strong economic influence, but caffeine has also infiltrated the soft beverage, pre-workout, and nootropics spheres. Coffee is frequently an aid for socialization (especially with rise to "the third place") and has established itself as a beneficial additive within many consumables. Understanding the impact of such a commonly consumed drug and its benefits/detriments to our cell function will help researchers predict future public health issues and gain more awareness on its usability.

In vitro studies are limited in their direct application to organisms because the cells are outside of their niche. The caffeine that was applied to the HT-29 cells is not subject to factors like pharmacokinetics, systemic metabolism, and tissue responses. However, to mimic the human niche, caffeine treatments followed the average half-life of 5 hours [6]. The choices for concentrations of caffeine treatments in preliminary and future studies are based on both the average consumption of caffeine in college students, 159 mg [7], and on the EFSA's risk assessment for caffeine, which found 400 mg to be safe with habitual use in non-pregnant people [8]. The conversion of 159 mg and 400 mg of caffeine to molar values are 0.82 mM and 2 mM respectively. To stay below a 2 mM (~400 mg) range, 0.1 mM and 0.25 mM were chosen because the concentrations have practical food applications that are commonly consumed. For example, 0.1 mM equates to 19 mg of caffeine which is roughly the caffeine content in a chocolate bar, 0.25 mM is around 49 mg of caffeine which can be found in a can of cola, 0.82

mM is about 159 mg of caffeine which is equivalent to two shots of espresso, and 2 mM is 388 mg of caffeine and equals 4 cups of coffee [9].

One method for assessing the potential cytotoxic effects of caffeine was looking for an IC50 via an Alamar Blue assay. An IC50 is a measurement of the concentration of a drug that is needed to produce an inhibitory effect by 50%. Alamar Blue is a supravital stain that visualizes cell metabolism via fluorescence and can help to determine an IC50 value for a drug. Resazurin is the chemical in the stain that acts as a redox reaction indicator in a reaction with NADH. Reduction of resazurin leads to structural change to a pink fluorescent molecule called resorufin. Because NADH is a product of metabolism, the change from a nonfluorescent blue color to a fluorescent pink can be used as a measure of cell viability to calculate the IC50 of a drug.

A second method for evaluating the potential cytotoxic effects of caffeine was measured by assessing cell viability using a Trypan blue assay. Cell viability measures the proportion of healthy cells in a cell population, which is affected by environmental pressures, which can include toxins and drugs. Trypan blue is a vital stain that is only taken up by cells with damaged membranes, so a blue cell indicates that the cell is dead or dying. Viability and dead cell percentages can be calculated by counting the number of blue or dead cells and comparing them to the non-blue, or viable, cells.

Quantitative polymerase chain reaction (qPCR) is a molecular method that was used to quantify gene expression for chosen genes based on results from the cell viability and IC50 assays. The molecular method uses the amplification process from traditional PCR to quantify the genes in question by measuring the fluorescence of labeled nucleotides with each cycle. A relative qPCR method was used to compare differences in gene expression between the treated and untreated cells using a reference gene—like GAPDH or GSS—to build a standard curve that is used to normalize target gene sequences for quantification.

Preliminary Studies

Alamar Blue

The Alamar Blue assay was carried out using a 96-well plate of confluent HT-29 cells [10]. The cells were treated with four varying concentrations of caffeine. Treatments of 150 µl for each well were created using the ratios of 10 mM caffeine stock solution and McCoy's media found in Table 1. The media was removed using a single channel pipette before the treatments were applied to incubate at 37°C for 5 hours.

The treatments were removed after five hours, and the control well treatments as shown in Table 1, were applied and allowed to incubate for 10 minutes. All the treatments were then removed, and100 µl of McCoy's media and 20 µl of Alamar Blue solution were added to each well. The 96-well plate was gently rocked to mix the dye solution over the cells and then incubated in the 37°C CO_2 incubator for one hour. The plates were then read using a Synergy LX plate reader to measure fluorescence, with excitation settings at 530/25 nm and emission at 590/35 nm.

The calculations for the graphs in Figure 1A and 1B were set and graphed in Excel based on the raw data collected by the plate reader. The raw relative fluorescence unit (Raw RFU) was calculated by averaging all florescence units in wells with the same caffeine concentration. To calculate the percentage of fluorescence, the formula below was utilized:

% Fluorescence = (Raw RFU/Raw RFU Control) * 100

While initially hypothesizing an $IC_{50,}$ the data corresponded to an $EC_{50.}$ To calculate $EC_{50,}$ the slope-intercept form of a linear equation with Y equaling 50 was utilized. Slope (m) was calculated by Excel and "X" was solved to represent EC_{50}.

Table 1. 96-Well Plate with HT-29 cells and Corresponding Treatment Plan

	1	2	3	4	5	6	7	8	9	10	11	12
	Neg Ctrl	Neg Ctrl	0.1 mM	0.1 mM	0.25 mM	0.25 mM	0.82 mM	0.82 mM	2 mM	2 mM	50% EtOH	No cells
Caff. *	0 µl	0 µl	1.5 µl	1.5 µl	3.75 µl	3.75 µl	12.3 µl	12.3 µl	30 µl	30 µl	75 µl EtOH *	0 µl
Media	150 µl	150 µl	148.5 µl	148.5 µl	148.5 µl	148.5 µl	146.25 µl	146.25 µl	120 µl	120 µl	75 µl	0 µl

Results

An Alamar blue assay is used to visualize the number of cells which are metabolically active, indicating cell survival. This assay can determine cell viability and survival in response to varying concentrations of caffeine. That information can predict the IC_{50} of caffeine and provide direction for MRE'SS final semester project.

With % fluorescence being a good indicator for cell viability and activity in Alamar Blue assays, the initial incubation period of one hour showed a slight decrease in cell metabolic activity as the caffeine treatment concentration increased. This one-hour assay indicates a slight inverse correlation between caffeine concentration and metabolic activity (Fig. 1A). The inhibitory characteristics of caffeine don't show significant cytotoxic factors up to a concentration of 2 mM,

as the fluorescence % of the cells only decreased by 5% from 100% to ~95% (Fig. 1A). IC50 cannot be calculated because the assay did not exhibit a 50% inhibition of cellular metabolism.

Surprisingly, the Alamar Blue Assay that incubated overnight for 17 hours showed contradicting results to the one-hour Alamar Blue assay. The % fluorescence sharply increased as caffeine concentration increased from 0 mM to 0.25 mM. A continued gradual increase and plateau is observed between 0.25 mM and 0.82 mM until a decrease in % fluorescence is observed only after 0.82 mM (Fig. 1B). A 0.82 mM caffeine concentration shows the greatest influence on increasing cellular metabolic activity, with % fluorescence peaking at 275%. A EC50 of 0.04 mM can be estimated from Fig. 1B.

This shift in cellular activity under the treatment of caffeine redirects the initial experimental objective of determining caffeine's cytotoxicity range, to determining caffeine's metabolic enhancement range or influence on metabolic activity.

The observed difference within both Alamar Blue assays was likely due to the increased incubation time allowing the HT-29 cells to properly follow through with the cellular responses, as opposed to the one-hour incubation time which only allowed for a limited perspective on the HT-29 cells response to caffeine. An overnight incubation period gives HT-29 cells time to recover from possible stress responses that initially caused decreased metabolic activity, and opportunity to reduce more resazurin in the Alamar Blue.

The significant increase of metabolic activity in lower caffeine concentrations, followed by a decrease observed in higher caffeine concentrations (Fig. 1B) may also be a good representation of caffeine's biphasic nature. The increasing % fluorescence at lower concentrations may be caffeine increasing general metabolic activity or inducing a stress response that increases metabolic activity. The decrease in % fluorescence as caffeine concentration increases above 0.82 mM could be due to an induced stress response that the HT-29 cells cannot recover from, or induced cytotoxicity.

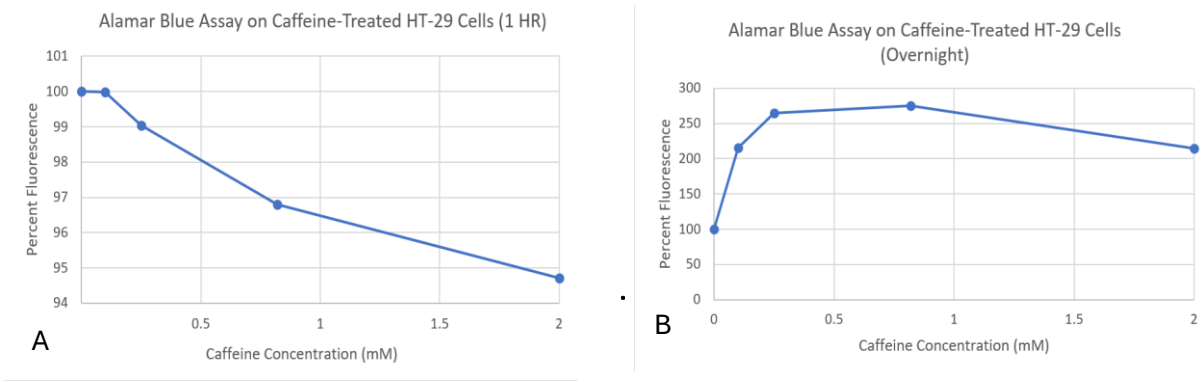

Figure 1. Fluorescence data of Alamar assay using HT-29 cells treated with varying caffeine concentrations. Data was taken after (A) 1 hour and (B) 17 hours of incubation. The data represents the metabolic activity of cells treated with 0 mM (negative control), 0.1 mM, 0.25 mM, 0.82 mM, and 2 mM of caffeine for 5 hours. Positive control (50% ethanol) not visualized here. EC50 measured at 0.04 mM caffeine.

Trypan Blue

The Trypan Blue Assay was carried out using a 6-well plate of confluent HT-29 cells [10]. Treatments of 2 ml for each well were created using the ratios of 10 mM caffeine stock solution and McCoy's media found in Table 2. Media in treatment wells 2 through 4 was removed before the treatments were applied. The cells with the treatments were then set to incubate at 37°C for 5 hours. The control tests in wells 1 and 2 were carried out as shown in Table 2 and left to incubate for 15 minutes near the end of the caffeine treatment time. All of the treatments were then removed, and the cells were washed with 1 ml of PBS.

The aliquot of Trypan Blue dye was centrifuged at maximum speed to pellet the unwanted precipitate. Following this, the PBS wash was removed and another 500 µl of PBS was applied as well as 100 µl of the Trypan Blue from the top of the centrifuged tube with the stain. The 6-well plate was gently rocked to mix the dye solution over the cells and was allowed to sit for five minutes. Visualization of the assay was carried out with an inverted (true color) microscope and images of each well were captured, as shown in Figure 2.

The percent viability calculation for Figure 3 was calculated by counting the cells in one quarter of the image of each well taken from the inverted (true color) microscope. The value was multiplied by four to obtain the total estimated cell count. Next, the blue cells were counted and subtracted from the estimated total cells, and that value was divided by total cells and multiplied by 100 to get percent viability.

Table 2. 6-Well Plate with HT-29 cells and Corresponding Treatment Plan

W1: No treatment- Negative Control 10 mM caffeine: 0 µl MEDIA: 2 000 µl	**W2: 50% ethanol treatment – Positive control** 200 proof ethanol: 1000 µl MEDIA: 1000 µl	**W3: 0.1 mM caffeine** 10 mM caffeine: 20 µl MEDIA: 1980 µl
W4: 0.25 mM caffeine 10 mM caffeine: 50 µl MEDIA: 1950 µl	**W5: 0.82 mM caffeine** 10 mM caffeine: 164 µl MEDIA: 1836 µl	**W6: 2 mM caffeine** 10 mM caffeine: 400 µl MEDIA: 1600 µl

Results

The Trypan Blue assay uses a vital stain to visualize the number of cells which have damaged membranes within the varied caffeine solutions. Figure 3 shows the percent viability of the HT-29 cells for each of the treatment wells. Although there was a slight decrease in viability with increasing caffeine concentration, the viability of the highest concentration, 2 mM, was still relatively high at 92%.

Unlike the Alamar Blue results, there is no indication of potential EC_{50} based on Figure 3. This is likely due to the Trypan results being read immediately after treatment, whereas the Alamar results were reread after 17 hours. The cells in the 6-well plate did not have time to recuperate from the potential stress response induced by caffeine, which is why some of the cells died.

Figure 2 shows the physical observation of cell morphology and stain uptake after Trypan Blue was given to the cells. Although there were some cells that had taken up the stain, it appears that most of the cells were unaffected in a terminal sense. A future experiment with caffeine-treated HT-29 cell RNA will examine the molecular effects that the drug has on the cells. For now, these treatment concentrations, which line up with commonly consumed foods mentioned in the proposal for this experiment [9], do not necessarily indicate a cytotoxic effect.

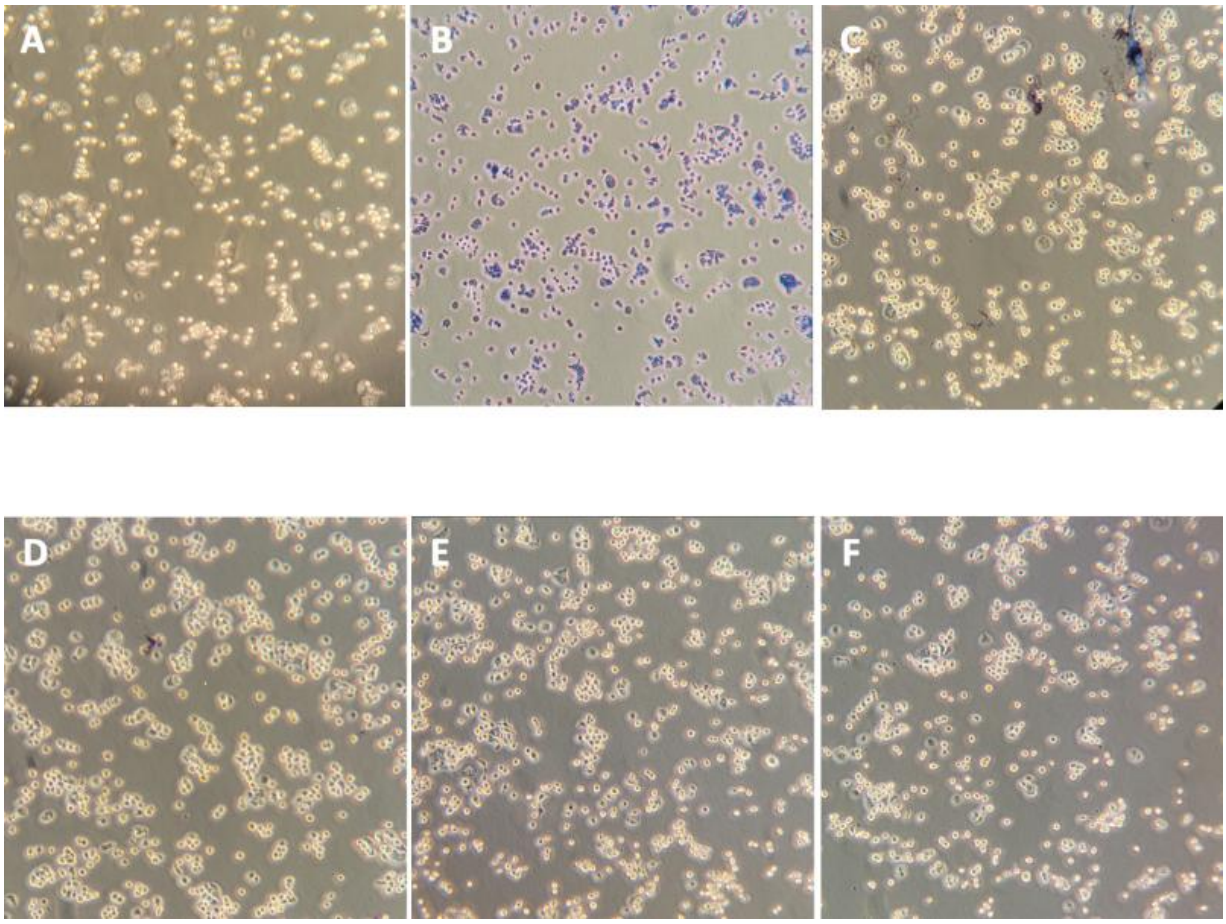

Figure 2. Stained results of 6-well Trypan Blue assay visualized with inverted phase contrast microscope. (A) Negative control, 100% cell viability. (B) Positive control with EtOH, 0% cell viability. (C) 0.1 mM caffeine treatment, 97.6 % cell viability. (D) 0.25 mM caffeine treatment, 97.3% viability. (E) 0.82 mM caffeine treatment, 93.5% cell viability. (F) 2mM caffeine treatment, 91.9% cell viability.

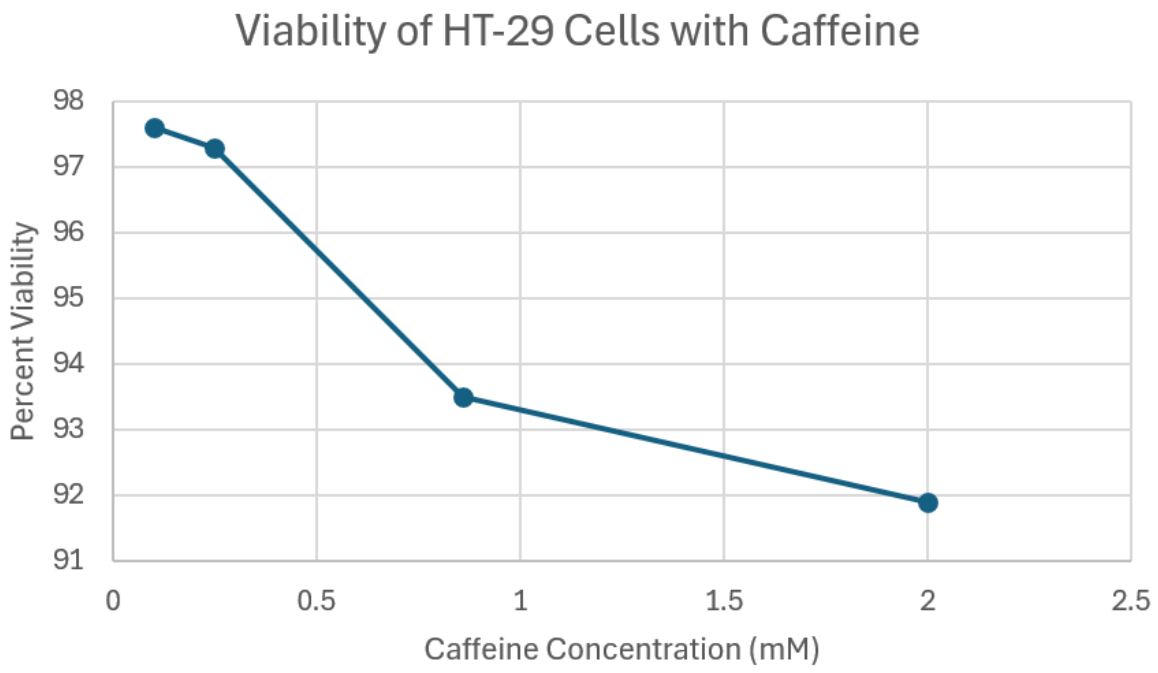

Figure 3. Graphed percent viability for HT-29 cells treated with caffeine concentrations 0.1, 0.25, 0.82, and 2 mM for 5 hours. The negative and positive controls (not visualized here) had 100% and 0% cell viability, respectively. Viability was determined using Trypan Blue assay to compare dead to living cells.

QPCR

The Trypan and Alamar blue cytotoxicity assays showed that the caffeine concentrations used on the HT-29 cells (0.1, 0.25, 0.82, and 2 mM) had less of an inhibitory effect on the cells but instead increased cellular metabolism. The Trypan blue results showed a cellular viability that remained above 91% and the Alamar blue results showed an EC_{50} of 0.04 mM for caffeine [10].

Although previous studies have shown that caffeine can induce apoptosis and cytotoxicity in various cancer cell lines including glioma and gastric cancer cells [4, 5], our experiment revealed increased metabolic activity in caffeine-treated HT-29 cells. These unexpected increases in metabolic activity prompted MRE'SS to investigate gene expression changes related to cell stress and inflammation, apoptosis, and proliferation using the following genes:

- IL8: inflammation-related cytokines
- BCL2: anti-apoptotic regulator
- RPL5: ribosomal protein, associated with proliferation
- RHOA: small GTPase involved in cytoskeletal regulation and motility

- CASP3: executioner caspase critical in apoptosis

By analyzing the expression patterns of these genes, MRE'SS aimed to determine what molecular changes might account for the enhanced metabolism observed in response to caffeine treatment.

Table 3. qPCR Sample Run Order

Rotor spot	Identifier (Condition-Gene-Dilution)
1	U-Gapdh-1:10
2	U-Gapdh-1:100
3	U-Gapdh-1:1000
4	U-Gapdh-1:10000
5	U-GSS-1:10
6	U-IL8-1:10
7	U-BCL2-1:10
8	U-RPL5-1:10
9	U-RHOA-1:10
10	U-CASP31:10
11	T-Gapdh-1:10
12	T-Gapdh-1:100
13	T-Gapdh-1:1000
14	T-Gapdh-1:10000
15	T-GSS-1:10
16	T-IL8-1:10
17	T-BCL2-1:10
18	T-RPL5-1:10
19	T-RHOA-1:10
20	T-CASP34-1:10
21	No Template Control
22	No RT Control
23 (Optional)	No RT Control (secondary)

**U = untreated, T = Treated*

Results

Figure 4 shows the standard curve produced by the qPCR run of the 0.82 mM caffeine-treated and untreated HT-29 cells. The curve's data indicates a correlation coefficient (R) of 0.78857, and a squared correlation coefficient value (R^2) of 0.5387. The efficiency value is 4.05378, or 405%.

A melting curve is meant to verify the specificity and purity of the amplified products in the qPCR run. Figure 2 contains the melting curve data for this qPCR run. The peaks represent the melting

temperatures for the PCR products. There are multiple peaks seen for a majority of the values in Figure 5. The control samples also contained peaks, and Figure 6 presents the raw melting curve data for the no template and no RT controls. The peaks seen at higher temperatures represent longer amplicons that form early on in the PCR process. Five of the samples had single peaks, which include Untreated 1:100 dilution GAPDH at 88.7 °C, untreated BCL2 at 80.0 °C, treated 1:10 dilution GAPDH at 86.5 °C, treated RPL5 at 89.0 °C, and RHOA at 81.2 °C.

Table 4 presents the summary of RT-qPCR for Caffeine-Treated HT-29 cells. Only 3 genes of interest (IL8, BCL2, and RHOA) were capable of being evaluated because two of them (RPL5 and CASP3) were missing Ct values from the quantitation report. To determine the fold difference, the expressions were normalized against the 1:10 GAPDH Ct values with the equation:

$$2^{(Ct\,(GAPDH)-Ct\,(GENE))}$$

For fold difference values less than one, the expression 1/ (fold difference) was used to determine the fold decrease. IL8 had a 4-fold decrease in expression, BCL2 had a 1.2-fold increase, and RHOA saw a 40-fold decrease in expression.

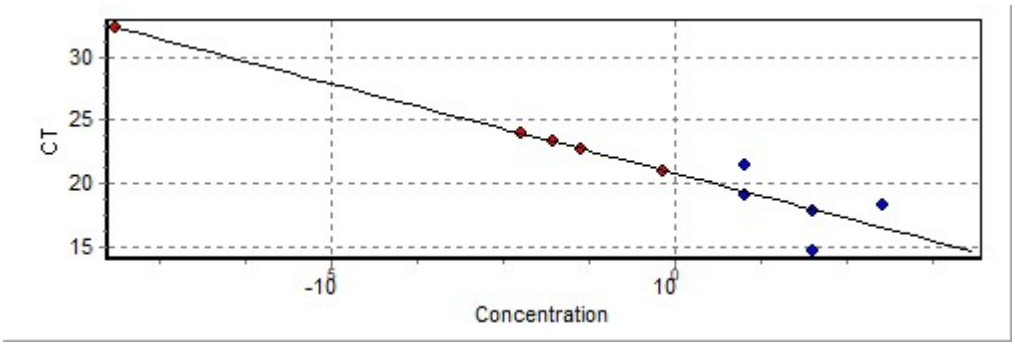

Figure 4. Standard curve of qPCR data for selecting genes in caffeine-treated and untreated HT-29 cells. This standard curve generated from a dilution series of GAPDH, shows an R^2 = 0.5387 and an efficiency value of 405%.

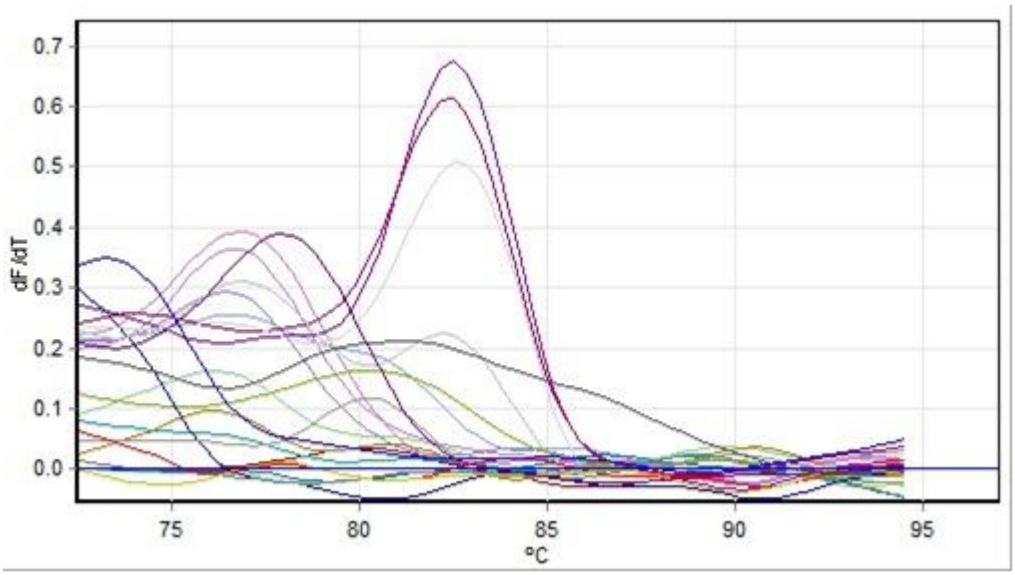

Figure 5. Melting curve of qPCR data for caffeine-treated and untreated HT-29 cells. The melting curve data showed multiple peaks in most samples. Few samples, such as BCL2 and RHOA showed single peaks.

No.	Color	Name	Genotype	Peak 1	Peak 2	Peak 3	Peak 4
66		No Template Control		77.8	93.3		
67		No RT Control		73.3	85.0		
68		No RT Control (secondary)		78.0			

Figure 6. Raw melting curve data for controls in qPCR reaction for HT-29 cells**.** No template controls and no-RT controls displayed multiple melting peaks, with No Template Control peaking between 77-94°C and No RT Control peaking between 73-85°C.

Table 4. Summary of RT-qPCR for Caffeine-Treated and Untreated HT-29 Cells

Values	GAPDH	IL8	BCL2	RPL5	RHOA	CASP3
Untreated Ct	17.74	25.14	35.69	DA*	21.00	DA*
Treated Ct	14.62	23.96	32.33	DA*	23.29	DA*
Untreated Normalized Expression	-	5.9×10^{-3}	3.9×10^{-6}	-	1.0×10^{-1}	-
Treated Normalized Expression	-	1.5×10^{-3}	4.7×10^{-6}	-	2.5×10^{-3}	-
Fold difference	-	0.25	1.2	-	0.025	-

Normalized expressions calculated with $2^{(Ct\ (GAPDH)-Ct\ (GENE))}$. Fold difference calculated as (treated normalized expression value)/(untreated normalized expression value).
**DA = data unavailable for gene's Ct value.* For values <1, reciprocal is used to express fold decrease.

Caution was taken when examining the qPCR data to draw conclusions. The standard curve (Fig. 4) has questionable data, as indicated by the low squared correlation coefficient value (R2) of 0.5387. A good correlation value is 0.98 or greater. Also, the efficiency value is too high at 405%. Normally, a good efficiency value should fall between 80% and 100%. The likely causes of poor standard curve data could be due to problems with primers, pipetting errors with dilutions, and issues with the sample quality. The melting curve (Fig. 5) also indicates issues with the experiment due to the multiple peaks, which suggest non-specific products, primer-dimers, and contamination. Future qPCR investigation should use more caution to avoid these issues.

Putative Conclusions

The cytotoxicity experiments via the Alamar and Trypan Blue assays provided data that leads to a revised interpretation of caffeine's effects. The research done going into the experiments led to conclusions that caffeine negatively affects cells, but the results of MRE'SS exploration of caffeine on HT-29 cells provided evidence of increased cellular metabolism [10]. This is supported by the Alamar Blue data of an EC50 at 0.04 mM rather than an IC50. One difference to consider between MRE'SS undergraduate research and that carried out by professional researchers is that of caffeine concentration in experiments. Preliminary research by MRE'SS found studies that used concentrations of 1 mM, 2 mM, 4 mM, and 8 mM [4, 5]. Perhaps the type of response to caffeine depends on the dosage, where lower concentrations result in increased metabolism and higher dosages lead to cytotoxic results.

A suspected mechanism behind these results is a caffeine-induced stress response to a nonlethal assault. Evidence for stress could be interpreted from the Trypan results with cell viability. While there was a decline in cell viability, it remained >90% across all caffeine treatments. This suggests a nonlethal assault by the caffeine. The increased metabolic activity seen with Alamar fluorescence may indicate increased ATP production and protein synthesis to mitigate the damage caused by caffeine.

Keeping in mind the caution of the qPCR interpretation, three genes had data that could provide some evidence towards a molecular response to the drug. Downregulation of IL8 and RHOA (a 4-fold and 40-fold decrease, respectively) in conjunction with upregulation of BCL2, point to altered inflammatory, apoptotic, and cytoskeletal pathways. The 40-fold decrease in RHOA may also indicate changes in motility or cytoskeletal dynamics in response to stress.

TEM analysis may provide further support for the effects of caffeine examined through the three experiments discussed above by providing a visualization of the structural changes to the cell after caffeine exposure.

Hypothesis

If increased cellular metabolism is due to cellular stress, then there will be structural signs of stress that can be visualized.

Specific Aim

Determine the occurrence of caffeine-induced cellular stress mechanisms through observing/identifying intracellular structural signs of stress. This aim is to test that increased cellular metabolism in caffeine-treated HT-29 cells are due to activated cellular stress mechanisms. Structural changes such as granulation, vacuolation, and swollen organelles like mitochondria and ER can point to stress mechanisms.

Research Strategy

Rationale

Initially, MRE'SS set out to investigate the cytotoxic effect of caffeine on colon cancer cells (HT-29) due to the increase in colorectal cancer in younger individuals and the drug's potential therapeutic usage [1,2]. There was also an interest due to the prevalence of caffeine usage in college students. Investigating the possible cytotoxic effects of caffeine was meant to shed light on the detrimental or beneficial effects of the drug for future public health research.

Cytotoxicity studies of caffeine on cells carried out by professional researchers used concentrations of 0.5, 1, 2, 4, and 8 mM of caffeine [4, 5]. MRE'SS used the concentrations 0.1, 0.25, 0.82, and 2 mM, which equated to commonly consumed foods, such as a chocolate bar, soda, two shots of espresso, and four cups of coffee [9, 10]. The lower concentrations used by MRE'SS pertained to real life caffeine consumption, which was meant to look for potential effects that might impact caffeine consumers.

The initial protocol for caffeine treatment in the MRE'SS Alamar Blue assay was 5 hours to mimic the half-life of caffeine in humans, followed by an hour of incubation with the Alamar dye. However, the fluorescence reading of the Alamar results did not show much metabolic activity for cells after the hour-long incubation. Dr. Johnson let the cells incubate overnight for a second fluorescence reading 17 hours post-treatment. The second reading was significantly different from the initial reading—it showed a metabolically enhancing response to the caffeine treatment. These results varied from the anticipated results of cytotoxicity to determine $IC_{50.}$ Instead, the second Alamar fluorescence reading determined an EC_{50} of 0.04 mM.

What are the cells experiencing that would cause the increased metabolic activity? Perhaps the caffeine concentrations used were not strong enough to kill the cells but instead caused enough damage to induce a stress response. Certain stress responses lead to increased metabolic activity to mitigate the damage caused by the drug. Under stress, cells activate repair mechanisms like protein refolding and increased mitochondrial activity, which raise ATP demand and overall metabolism to maintain homeostasis. A detailed look at the cells using transmission electron microscopy would show detailed structural changes within the cells, indicating the type of cell response. Stressed cells show granules and vacuolization, causing increased compartmentalization. Morphological changes can also be observed in the mitochondria and endoplasmic reticulum.

Transmission electron microscopy is a method that members of MRE'SS have not carried out before due to undergraduate status. Samples require special preparation before visualization with electrons can occur. To help develop a protocol for the experiment, the materials and methods will be based on the TEM methodology for cultured cell sample preparation shared by Lam, as well as a methodology by Wang et al. [11, 12]. Together, Lam's Electron Microscope Sample Preparation Technique and Wang et al's methodologies provide inexperienced electron microscopy undergraduate students with a framework that researchers in cell biology use with TEM technology.

With a revised understanding of the caffeine-treated HT-29 cells, a new procedural hypothesis is established. If increased cellular metabolism in caffeine-treated HT-29 cells is caused by induced cellular stress responses, then there will be structural signs of stress that can be visualized with TEM.

Materials & Methods

Secondary Cell Culture

HT 29 cells will be subcultured into 6 separate T25 flasks that correspond to the control or treatment concentration—positive, negative, 0.1 mM, 0.25 mM, 0.82 mM, and 2 mM, respectively. The cells will be cultured in 5 mL of McCoy's media and placed in a 37 °C incubator for 3 days to form a confluent monolayer.

Caffeine Treatment

A 25 mL stock concentration of 10 mM created with 48.6 mg of Sigma C0750 caffeine and PBS will be used to create the treatment concentrations. See Table 1 for the ratio of stock caffeine to McCoy's media for the different treatments. The HT-29 cells with their treatments will be placed in a 37 °C incubator for 5 hours. Post treatment, the control and treatment solutions will be removed, and the cells will undergo another incubation of 17 hours with 0.5 mL Alamar Blue

dye and 5 mL McCoy's media. The 17-hour length of time was chosen with the purpose of replicating the previously performed Alamar Blue assay, and its observed increased metabolic activity results. The negative control will receive only 5 mL of McCoy's media, and the positive control will receive a 50% ethanol treatment made with 2.5 mL of 200 proof ethanol and 2.5 mL of McCoy's. The length of treatment for both controls will be 15 minutes as per the prior Alamar Blue assay.

Table 5. Caffeine Treatment Preparation Volumes for HT-29 Cells in T25 Flasks

Treatment	Volume of 10 mM Stock (µL)	Volume of McCoy's Media (µL)	Volume of 200 proof EtOH*
Negative Control	-	5,000	-
Positive Control	-	2,500	2,500
0.1 mM	50	4,950	-
0.25 mM	125	4,875	-
0.82 mM	410	4,590	-
2 mM	1,000	4,000	-

**EtOH = Ethanol*

Transmission Electron Microscopy

Following treatment, each set of HT-29 cell cultures will undergo eight steps for TEM preparation based on Lam's protocol: fixation, en block staining, agarose embedding, dehydration, infiltration, resin embedding, resin-embedded sample trimming, and ultrathin sectioning [11]. Tables 2 and 3 list the reagents and equipment that will be needed for the preparation stage.

Table 6. List of Reagents for Transmission Electron Sample Preparation of HT-29 Cells

MilliQ water	Uranyl acetate
0.2 M Phosphate buffer (pH 7.4)	Lead citrate
0.2 M sodium-cacodylate (pH 7.4)	Agarose, low-gelling point
16% Paraformaldehyde solution, EM grade (PFM)	70, 80, 90, 95, and 100% Ethanol
	Propylene oxide
25% Glutaraldehyde solution, EM grade	Dodecenylsuccinic anhydride (DDSA)
2% osmium tetroxide	Araldite 502
Potassium ferricyanide	Procure 812
	Benzyldimethylamine (BDMA)

Based on Lam's protocol [11]

Table 7. List of Equipment for Transmission Electron Sample Preparation of HT-29 Cells

BioWave Pro microwave	Centrifuge
Cell scraper	Ultra UCT ultramicrotome with glass knife
0.22 µm Centrifuge Tubes	Ultra 45° Diatome with diamond knife

Based on Lam's protocol [11]

The treated and control HT-29 cells will be fixed by aspirating the treatment media and adding pre-warmed phosphate-buffered 4% PFM to sit on the cells at room temperature. After 1 hour, the cells will be washed twice with 0.1 M sodium-cacodylate. This step will be followed by post-fixing the sample with a 0.1 M sodium-cacodylate buffer and 2.5% glutaraldehyde for 24 hours at a temperature of 4 °C. Cells are then washed with a 0.1 M sodium-cacodylate buffer three times before post-fixing the sample with 1% osmium tetroxide and 1.5% potassium ferricyanide in a 0.1M sodium-cacodylate buffer on ice for an hour. The samples will then be exposed to three BioWave Pro microwave (Pelco) cycles of 120 seconds on and 120 seconds off at 100 watts under a vacuum. The final fixation step will be to rinse each sample three times with MilliQ water.

The en bloc staining step will involve adding 2% (w/v) uranyl acetate in MilliQ water to the sample with another three-cycle microwave exposure at 100 watts under a vacuum for 120 seconds on and 120 seconds off. The uranyl acetate will be filtered with a 0.22 µm pore filter, followed by another three rinses with MilliQ water. The agarose embedding step of preparation will result in a cell pellet. Using a cell scraper, the cells will be put into a centrifuge tube with MilliQ water and run in the centrifuge at 10,000 g for three minutes. The pellet will be resuspended in 70% ethanol and centrifuged again for 3 minutes at 10,000g. After the ethanol is removed, one drop of 100 °C agarose will be added to the pellet, vortexed, and centrifuged immediately. The final step of the en bloc stage will be to cut the solidified cell-agarose pellets into 1 mm cubes.

The next two steps will be to dehydrate and infiltrate the cell cubes. Dehydration will be done by using a graduated ethanol series of 80, 90, 95, 100, and 100% (v/v) in the microwave at 150 watts for 40 seconds each cycle. Propylene oxide of 100% and 100% (v/v) cycles in the microwave at 150 watts for 40 seconds will finish the dehydration step. Propylene oxide samples in a graduated series of 25, 50, 75, 100, and 100% (v/v) will then each be infiltrated by Resin made with 51.7% DDSA, 18.6% (w/w) Araldite 502, 26.3% (w/w) Procure 812, and 3.4% (w/w) BDMA at 250 watts for 180 seconds under a vacuum.

The dehydrated and infiltrated cell cubes will undergo resin embedding by putting the cubes into resin-block molds and filling them with resin. These will be incubated in a 60 °C oven for 48 hours while the resin polymerizes. The polymerized resin cell cubes will be trimmed with a razor blade to expose the cell pellet cubes, and a mirror surface will be created on the cubes using an Ultra UCT ultramicrotome (Leica Biosystems) that has a glass knife.

The final step to prepare the cell samples for TEM visualization will be to create ultrathin sections for viewing. 70 to 90 nm sections will be cut using an ultramicrotome that is equipped with a diamond knife (Ultra 45° Diatome) at an angle of 6°. The ultrathin sections will be loaded onto copper mesh grids with the copper side up. 2% (w/v) uranyl acetate and Reynolds' Lead Citrate

will stain the grids for 5 minutes and 3 minutes, respectively. The samples will then be ready for TEM imaging.

Prepared grids will be imaged with a JEOL JEM-1011 transmission electron microscope (JEOL Ltd.) with a voltage of 80 kV. Images will be captured to assess detailed subcellular structures for signs of cellular stress.

Anticipated Outcomes

The anticipated outcome that MRE'SS expects to see with transmission electron microscopy of caffeine treated HT-29 cells is structural signs of cell stress. The hypothesis is that the increased metabolic activity is due to a stress response from caffeine exposure. While the concentrations were lower than other cytotoxic studies [4, 5], there may have been nonlethal cellular damage. As a response, the cells needed to fix the possible damage which would involve increased need for ATP production, hence the increased fluorescence seen with the overnight Alamar data.

If cellular stress is the root cause of the metabolic activity, a look at the structural changes in the cells would likely show vacuolation, granulation, or mitochondrial and endoplasmic reticular swelling. Figure 7 shows examples of TEM images that exhibit cellular stress which were taken from Wang et al.'s look at ER and mitochondrial stress. The stressed cells had a swollen ER and mitochondria (Fig. 7B-D, F-H) compared to normal mitochondria and ER (Fig. 7A, E) [12].

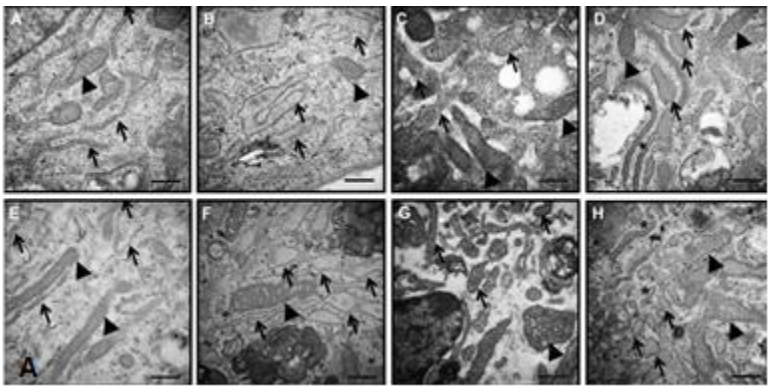

Figure 7. Examples of anticipated TEM results based on cell stress. (B-D, F-H) TEM images from Wang et al. that show examples of cellular stress through swollen mitochondria (arrowheads) and ER (arrows) compared to (A, E) normal mitochondria and normal ER [12].

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