International Baccalaureate

Biology Extended Essay

Research Question:

"How do varying concentrations of 98,5% pure caffeine (2, 4, 8, 10 and 20 mg/mL) affect the cell viability (cellular metabolic activity) of MCF-7 breast cancer cells after 24 and 48 hours of exposure, as measured by the MTT assay?"

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Introduction

Breast cancer has been one of the most prevalent cancers affecting all women worldwide, with different subtypes which differ in prognosis, treatment responsiveness, and biological behaviour. Being one of the most important issues of the public health throughout the world and in Türkiye, the incidence rates of breast cancer has been rapidly increasing due to the growing old aged population, changes in life style and availability of better diagnostic tools. In 2024 considered alone, approximately 2.3 million women were diagnosed with breast cancer and additionally, 670,000 women died from breast cancer disease globally [1]. Resulting from the rapid increase rates of breast cancer, this specific type of cancer has now been stated as the most commonly diagnosed cancer in women [2]. These subtypes, MCF-7 cells -an estrogen receptor-positive (ER+) human breast cancer cell line- serve as a well established vitro model that can easily be used for investigating molecular mechanisms of tumor growth and potential therapeutic agents.

Caffeine, a widely and frequently consumed psychoactive compound found in popular beverages such as coffee, tea and energy drinks has gained interest in cancer research due to its differing biochemical effects on human body. According to the International Coffee Organisation (2023) the estimated coffee consumption is around 175 million 60-kg bags yearly, which is equivalent to millions of daily servings worldwide [3]. While caffeine is best known and used for its intense mechanism of stimulating the human central nervous system, approaching studies suggest that caffeine is not only an innocent energy supply but it may also influence cellular proliferation-known as cancer-, apoptosis and oxidative stress pathways. Noticeably, it's ongoing potential role in modulating tumor cell viability has

triggered investigations looking depply into its anticancer properties. The National Institutes of Health (NIH 2024) suggests that a moderate consumption of caffeine doesn't seem to have a significant effect on cell viability however, a significant number of studies state that coffee consumption may reduse cancer risk [4].

Background Information

Caffeine has been reported to destabilize checkpoint control and block DNA damage repair pathways (including ATR/Chk1), causing cells with unrepairable DNA damage to proceed with the cell cycle and induce apoptosis, and also cellular oxidative stress, which can also play a role in diminished cellular viability in cancer cells.[4]

The toxic effects of caffeine on cancer cells appear to be dose and time dependant, often mediated by the generation of reactive oxygen species (ROS) and the disruptions in mitochondrial function. Prior studies such as the Rosendahl et al., 2015 — "caffeine inhibits MCF-7 growth" which has indicated that caffeine significantly inhibited cancer cell prohibition and the study Funakoshi-Tago et al., 2020 — "caffeine alone shows no effect; only helps tamoxifen" stated that caffeine had no effect on cancer cells have reported arguments regarding caffeine's effects on cancer cells however, investigations and conclusions in the current literature remain inconclusive, specially regarding the effects of caffeine on ER+ breast cancer cells such as MCF-7 [5, 6].

In order to subsidize to this rapidly growing field, the main aim of this study is to investigate how different concentrations of caffeine (2, 4, 8, 10, 20mg/mL) affect the viability of MCF-7 breast cancer cells after 24 and 48 hours of consistent expousure. The methods MTT assay- a dependable calorimetric technique used too measure cellular metabolic activity- will be used

to quantify changes in cell viability. This study aims to provide necessary insight into the cytotoxic potential of caffeine followed by its possible therapeutic relevance in breast cancer treatment by analyzing and observing MCF-7 breast cancer cells. Given the global concern in the matter of breast cancer and the unclear effects of caffeine regarding this situation, investigating whether different concentrations of caffeine alter the MCF-7 cell line viability will be built on the research question:

"How do varying concentrations of 98,5% pure caffeine (2, 4, 8, 10 and 20 mg/mL) affect the cell viability (cellular metabolic activity) of MCF-7 breast cancer cells after 24 and 48 hours of exposure, as measured by the MTT assay?"

Hypothesis: It was predicted that the lower and moderate concentrations of caffeine (2, 4,mg/mL m would have minimal to no effect on cellular viability, whereas higher concentrations of caffeine ($\geq 8\ mg/mL$) would have a significant effect in decreasing the cell viability.

Method Development and Planning

1. **Determination of Caffein Concentration Range**

   The doses of caffeine (2, 4, 8, 10 and 20mg/mL) in this study were chosen to examine a dose-dependent effect within a biologically significant range. The two lower concentrations (2 and 4mg/mL) represent physiological concentrations naturally found in human plasma after moderate intake of caffeine and allow the evaluation of whether dietary levels of exposure can influence the viability of MCF-7 cells, or not[5]. The high concentrations (8, 10 and 20 mg/mL) were selected based on previous in vitro cancer studies, where supraphysiological doses are used to identify cytotoxic

thresholds associated with mitochondrial dysfunction and apoptosis[6]. The usage of various concentrations allowed detecting a hypothetical threshold effect and allowed giving a definite ground to the analysis of dose-and time- dependent variations in cell viability.

2. Choosing the MTT Assay Technique

The MTT assay was chosen as it is a simple, yet cost-effective and sensitive technique of determining the cell viability in vitro through the observation of cellular metabolic activity. In comparison with other approaches such as the Trypan Blue exclusion, which only present a direct cell count, MTT assay is an indicator of mitochondrial metabolic activity and, thus, it provides quantitative data, which can be statistically analyzed to provide insights into small changes in cell viability. Taking into consideration the fact that the hypothesized cytotoxic effects of caffeine can be associated with the problems of mitochondrial dysfunction, the MTT assay was specially suitable in this research.

Variables

Type of Variable	Description	Method of Control
Independent Variable	Caffeine dosage/concentration (mg/mL)	Six different caffeine concentration groups were prepared: 0 (control), 2, 4, 8, 10 and 20
Dependent Variable	MCF-7 Cell Viability	Determined by the usage of MTT Assay technique and calculated using the formula: Cell/mL = (Total live cells counted) x 2 (Dilution coefficient) x 104 (Total area factor) / Number of squares counted.

Controlled Variables	How is it contolled?	Why is it contolled?
Cell Type Used	Through all trials the MCF-7 Cell line was used.	Different cell types respond differently to caffeine; controlling cell type ensures results are specific to MCF-7 cells and comparable across treatments.
Cell Composition and Passaging	All cells were passaged within a constant passage range.	Variations in passaging number may alter growth rate of cells and drug sensitivity; controlling the range ensures the changes within cells are solely due to caffeine.
Environment	All cells were carried out in a laminar flow cabinet (Nüne MN 120) using environment and materials strlized with UVC Lamp.	Controlling environmental conditions prevents stress-induced effects on cell viability, since cellular metabolism is sensitive to environmental changes.

Trial Number/Replicates	All experiment group trials were replicated 8 times (n=8) to ensure statistical accuracy.	Ensures reliability and allows valid statistical resultings.
Cell Multiplication Method	Same seeding density and subculturing process for all wells.	Initial cell number variation has an effect on nutrient availability and growth, and viability is affected without caffeine intervention.
Viability Calculation Method	All cells in the area where the vertical and horizontal lines on the thoma slide intersected were counted using a light microscope and calculated using the formula = Viable cell count/total cell count x 100.	The application of a single method will be consistent and will not create variation due to differences in assay techniques.
Exposure time	Two exposure times were fixed (24 h and 48 h) for all experimental groups.	The cytotoxic effects are time dependent; the exposure time should be controlled to ascertain that the differences experienced are dose dependent.

Materials and Methodology

Material List

All materials and apparatus were sterile and suitable for cell culture.

Material	**Quantity**	**Uncertainty Unit**
RPMI-1640 culture medium	500 mL	**± 1 mL**
Fetal Bovine Serum (FBS)	100 mL	**± 0.5 mL**
L-Glutamine solution (200 mM)	10 mL	**± 0.1mL**
Penicilin-Streptomycin (10,000 U/mL)	10 mL	**± 0.1 mL**
HEPES buffer (1M)	10 mL	**± 0.1 mL**
Phosphate-buffered saline (PBS, pH 7.4)	100 mL	**± 1 mL**
Trypsin-EDTA (0.25%)	10 mL	**± 0.1 mL**
MTT solution (5mg/mL)	10 mL	**± 0.1 mL**
Caffeine stock solution	10 mg	**± 0.05 mg (prepared to 2-20 $\mu g/mL$)**
Dimethyl sulfoxide (DMSO)	5 mL	**± 0.05 mL**
MCF-7 cell suspension	1×10^4 cells	**± 0.1** $\times 10^4$ **cells**
T-75 flask	10	**-**
96-well microplates	1	**-**
Micropipettes $0.1 - 10\ \mu L,\ 10 - 100\ \mu L,\ 100 - 1000\ \mu L$	3	$\pm$ 0.02 μL, $\pm$ 0.2 μL, $\pm$ 1 μL
CO_2 incubator	1	$\pm$ 0.5℃, $\pm$ 0.2% CO_2
Microplate reader (570 nm)	1	± 0.005 absorbance units
PH buffer calibration	7.2-7.4	± 0.05
Inverted microscope	1	-
Caffeine		

Table M1. Materials used in experiment: Culture Equipment and Insturments

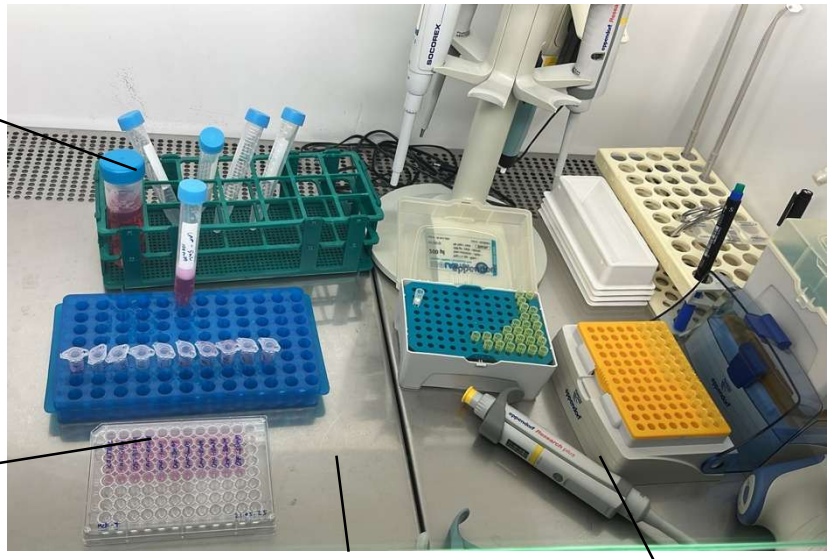


Figure 5: Laboratory Setup for MCF-7 Cell Treatment and MTT Assay

Methodology

A. Procurement of Cell Culture Line

MCF-7 (female breast adenocarcinoma) cells are a line obtained from human breast epithelium and are frequently used in breast cancer studies. The MCF-7 cell line was obtained from the Health Sciences University Gülhane Health Sciences Institute Stem Cell Research Center. The experimental processes of the study were performed in the Cell Culture Laboratory of the Physiology Department of Gülhane Medical Faculty. Since commercially purchased cell culture series was used in this study, it is not necessary to obtain ethics committee approval.

B. Multiplication of Cell Culture Line

1. MCF-7 cell line was carried out in a laminar flow cabinet (Nüve MN 120) using sterile environment and materials. Before and after the study, the UVC lamp inside the cabinet was operated for 15-20 min. and the ambient UVC lamps were operated for 1 hour.

2. MCF-7 cells were multiplied in DMEM medium (capricorn), 10% fetal bovine serum (FBS), 1% L-glutamine, 25 M HEPES and 1% penicillin and streptomycin antibiotics (PSA) containing medium (95% humidity, 37 °C and 5% CO2, incubator-phci panasonic).

3. For the thawing of cells; frozen cell suspension in cryovial tubes was removed from the liquid nitrogen tank in the Physiology Laboratory and kept in a water bath at 37 ºC. Then, cell medium was added to the freezing solution in the vial in a volume that would be twice as much as the freezing solution and transferred to a 15 mL sterile falcon tube. It was then centrifuged at 300 g for 3 min. After centrifugation, the cell pellet that settled to the bottom was seeded into 75 cm2 flasks to which 12 mL of cell medium was added.

C. Passaging of Cell Culture Series

1. The cell medium in the flask was changed every 72 hours. The cells examined under an inverted microscope (nikon eclipse TS100) were passaged with 0.25% trypsin EDTA solution when they filled 70-80% of the flask bottom.

2. 2 mL of trypsin-EDTA solution was added to 25 cm2 flasks and 4 mL of trypsin-EDTA solution was added to 75 cm2 flasks to remove cells adhering to the bottom.

3. After the medium of the flasks was removed, trypsin was added and left to incubate in the incubator for 1-2 minutes. It was quickly checked with an inverted microscope whether the cells had lifted from the surface and after it was determined that all cells had lifted from the bottom, cell medium twice the amount of trypsin was added.

4. The cell suspension in the flask was taken to a sterile falcon and centrifuged at 300 g for 3 minutes and then the supernatant was removed. The cells at the bottom were seeded at a number of ~7x105 cells in 5 mL of medium in 25 cm2 flasks, while the cells at a number of ~2.1x106 cells were seeded in 12 mL of medium in 75 cm2 flasks.

D. Determination of Viable Cell Count with Trypan Blue

1. Trypan blue powder was prepared by dissolving it in 0.4% phosphate buffer solution (PBS). The cell suspension and trypan blue solution were mixed in a ratio of (1:1) and 10 µL was taken and placed on the thoma slide.

3. All cells in the area where the vertical and horizontal lines on the thoma slide intersected were counted using a light microscope. It was determined that the membranes of the cells whose cytoplasm's were stained blue were broken and dead, while the viable cells were found to be transparent, maintained their membrane integrity and did not take the dye into their cytoplasm.

4. The percentage of viable cells (%) was calculated using the formula = Viable cell count/total cell count x 100. The experimental study was started at values where cell viability was 97% and above.

It was calculated using the formula Cell/mL = (Total live cells counted) x 2 (Dilution coefficient) x 104 (Total area factor) / Number of squares counted.

F. Determination of the Effect of Caffeine on 24 and 48-hour MCF-7 Cell Viability by MTT Method

1. The MTT (tetrazolium salt) method is a frequently used colorimetric test based on the measurement of metabolic activity used in the evaluation of cell proliferation, viability and cytotoxicity. With this method, the proportion of living cells in the cell population can be calculated quantitatively.
2. 24 and 48 hour dose studies were conducted by applying different concentrations of caffeine. The effect of caffeine doses (0, 2, 4, 8, 10, 20mg) on cell cytotoxicity was evaluated by the MTT method.

3. MCF-7 cells were seeded into a 96-well plate with a multichannel pipette in 8 replicates so that each well contained approximately 10×103 cells/100 mL cell medium for each experimental group. After the cells were incubated in the incubator for 24 hours, caffeine doses were applied to the wells according to the study plan and incubated for 24 and 48 hours. At the end of incubation, the solution on the cells was removed. 100 µL of MTT solution (5 mg/mL) was added to the groups and left to incubate for 2-4 hours in the incubator. At the end of incubation, the MTT solution was removed and 100 µL of dimethylsulfoxide (DMSO) was added to each well and after 20 min of incubation, the absorbance value of the wells at a wavelength of 570 nm was measured on the plate reader (Molecular Devices Filter Max F5) and the average absorbance value of each group was determined. The percentage viability of the cells was calculated as (Average of absorbance of caffeine-applied wells / Average of absorbance of control wells) x100

Ethical, Safety and Environmental Considerations

Ethical Considerations

1) The experiment was carried out in in-vitro (cell-based) environment and no experiment included the usage of a human being or animal therefore, approval from the ethical committee wasn't required/requested.
2) The experiment involved the usage of the MCF-7 Cell line which was obtained from a certified biomedical research facility (Gülhane Health Sciences University, Türkiye). The cell line was experimentally available and had been established for research purposes only, no samples were collected from humans.

Safety Concerns

1) All experiments were carried under a biosafety level 2 (BSL-2) labaratory with a Class II laminar flow hood in order to maintain sterility while protecting the person working in the labaratory. During all procedures, personal protective equipment including sterile gloves, a lab coat and protective googles were worn.

Environmental Considerations

1) DMSO and caffeine residues were heavily diluted and neutralized before disposal in order to protect water systems from chemicals.
2) All cell culture apparatus were sterilized after the experiment to prevent environmental contamination/waste.
3) Specifically MTT, a tetrazolium salt, was collected in biohazard containers rather than being disposed in sinks or general waste.

Data Collection

Raw quantitative data

Data Table 1: Raw Absorbance Values (570 nm) of MCF-7 Cells After 24 h of Caffeine Exposure

Raw absorbance measured at 570 nm (OD 570 nm)

Caffeine ($\mu g/mL$)	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8
0	2.3993	2.8847	2.5071	2.0277	2.8976	2.2542	2.0300	2.506
2	1.7596	2.0200	1.8480	1.8677	1.7446	1.5389	2.1295	1.3159
4	1.0075	1.5587	1.4561	1.8929	1.7451	0.9664	1.4016	1.2714
8	1.3242	1.0059	0.8319	1.1545	1.4002	1.0281	1.3942	0.7965
10	0.9730	0.8446	1.1954	0.9989	1.2558	1.0364	1.1707	0.7787
20	0.1878	0.2246	0.1062	0.3472	0.1159	0.4174	0.4012	0.2597

Each value represents mean optical density (OD 570 nm) measured per well after 24 h incubation.

Data Table 2: Raw Absorbance Values (570 nm) of MCF-7 Cells After 48 h of Caffeine Exposure

Caffeine ($\mu g/mL$)	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8
0	1.6919	1.66	1.921	2.2467	2.1484	2.1315	2.0242	1.6189
2	1.3738	1.8221	1.6481	1.7194	1.8095	1.564	1.4672	1.1164
4	1.483	1.4308	1.8238	1.8001	1.7927	1.6886	1.6728	1.013
8	0.9044	0.8097	1.1374	1.0944	0.8955	0.9541	1.0848	0.7311
10	0.7363	0.7202	0.737	0.7214	0.7266	0.7299	0.6709	0.7019
20	0.2314	0.1909	0.1923	0.1979	0.4224	0.2785	0.3507	0.3272

Absorbance measured at 570 nm indicates mitochondrial enzymatic activity proportional to viable cells.

Data Table 3: Raw Cell-Viability Percentage of MCF-7 Cells After 24 h of Caffeine Exposure

Caffeine ($\mu g/mL$)	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8
0	98.399	118.306	102.820	83.159	118.835	92.448	83.253	102.775
2	72.164	82.843	75.789	76.597	71.549	63.112	87.334	53.967
4	41.319	63.925	59.717	77.631	71.569	39.633	57.482	52.142
8	54.307	41.253	34.117	47.348	57.424	42.164	57.178	32.665
10	39.904	34.638	49.025	40.966	51.502	42.504	48.012	31.935
20	7.702	9.211	4.355	14.239	4.753	17.118	16.453	10.650

Percentage viability = (mean absorbance of treated wells / control) x 100.

Data Table 4: Raw Cell-Viability Percentages of MCF-7 Cells After 48h of Caffeine Exposure

Caffeine ($\mu g/mL$)	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8
0	87.648	85.996	99.517	116.390	111.297	110.422	104.863	83.867
2	71.169	94.393	85.179	89.073	93.740	81.023	76.008	57.835
4	76.826	74.122	94.481	93.254	92.870	87.578	86.660	52.478
8	46.852	41.946	58.923	56.695	46.391	49.427	56.198	37.874
10	38.143	37.309	38.180	37.371	37.641	37.812	34.755	36.361
20	11.987	9.889	9.962	10.252	21.882	14.427	18.167	16.950

All values rounded to two decimals for consistency.

Processed Data

The analysis of collected data was analyzed using IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL, USA) packet programme. The compatibility of data to normal distribution was evaluated with the Shapiro-Wilk test, whereas the homogeneity of the variations was assesed using the Levene test. One-way ANOVA was performed to determine the differences between groups in variables that conform to normal distribution. In the variables with significant differences as a result of ANOVA, Duncan multiple comparison test was used to determine which groups had differences. The significance level was claimed as $p < 0.05$ for all statistical tests. Outcomes were given as mean ± standard deviation (Mean ± SD).

Groups	**N**	**Absorbance (24 h)**	**Cell Persentage (24 h)**	**Absorbance (48 h)**	**Cell Persentage (48 h)**
Group-1 (control)	8	2.44±0.11	100.00±8.16	1.93±0.13	100.00±8.50
Group-2 (2)	8	1.78±0.09	72.92±7.35	1.57±0.11	81.08±6.93
Group-3 (4)	8	1.41±0.08	57.93±5.47	1.59±0.12	82.27±7.08
Group-4 (8)	8	1.12±0.10	45.81±6.02	0.95±0.10	49.29±6.24
Group-5 (10)	8	1.03±0.12	42.31±5.84	0.72±0.09	37.20±6.05
Group-6 (20)	8	0.26±0.05	10.56±3.21	0.27±0.05	14.19±3.54

Table.1 Descriptive Statistics $(Mean \pm SD)$ Based on 24 h and 48 h Measurements

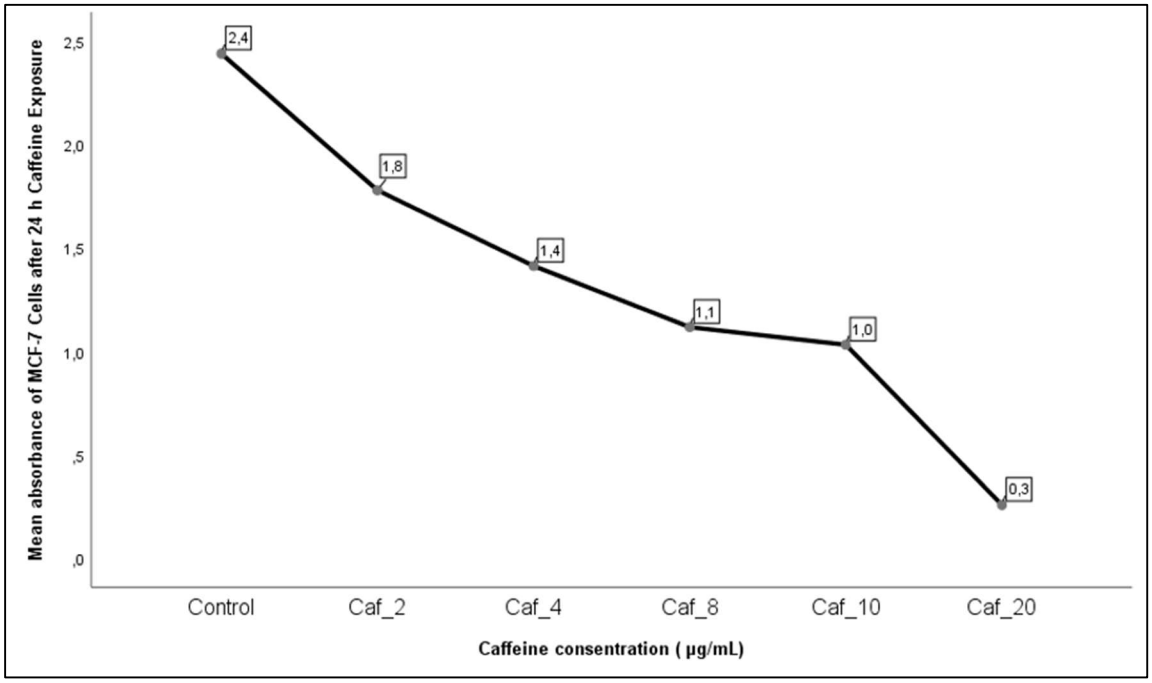
Table.2 Results of ANOVA and Duncan Tests

	F (5,42)	p	Duncan Grouping
Absorbance-24h	67.35	< 0.001	Gr-6 < Gr-5 = Gr-4 < Gr-3 < Gr-2 < Gr-1
Cell Viability-24h	67.35	< 0.001	Gr-6 < Gr-5 = Gr-4 < Gr-3 < Gr-2 < Gr-1
Absorbance-48h	84.81	< 0.001	Gr-6 < Gr-5 < Gr-4 < Gr-3 = Gr-2 < Gr-1
Cell Viability-48h	84.81	< 0.001	Gr-6 < Gr-5 < Gr-4 < Gr-3 = Gr-2 < Gr-1

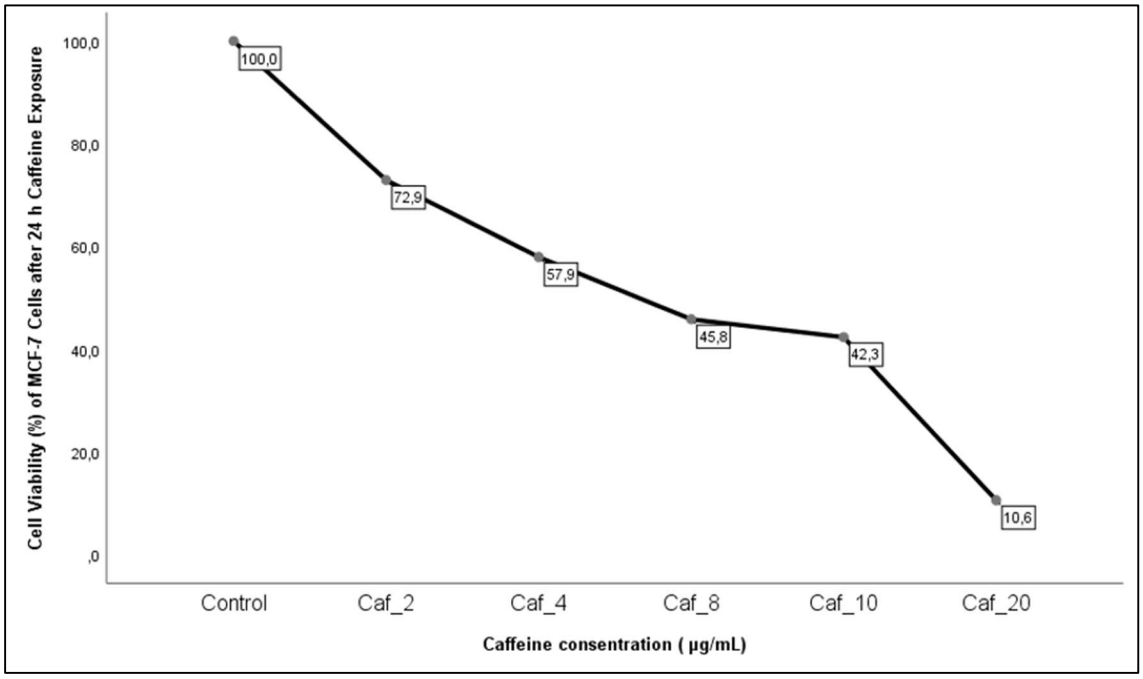
Outcomes

There were significant differences observed between the caffeine groups in terms of both absorbance and cell percentage values at both 24th and 48th hour measurements. (p0.001). According to the one-way variation analysis the data was found as; in the 24 hour measurements: F(5,42)=67.35, p0.001; in the 48 hour measurements: F(5,42)=84.81, p0.001. Duncan's multiple comparison test results indicated that the mean values for both time points increased significantly as caffeine concentration decreased. The means in the 24th hour were sorted as: Group 6< Group 5 = Group 4< Group 3< Group 2< Group 1. The means in the 48th hour were sorted as Group 6< Group 5< Group 4< Group 2 = Group 3< Group 1. These indications demonstrate that as the concentration of caffeine decreases, the quantities of both absorbance and percentage of cells significantly increases.

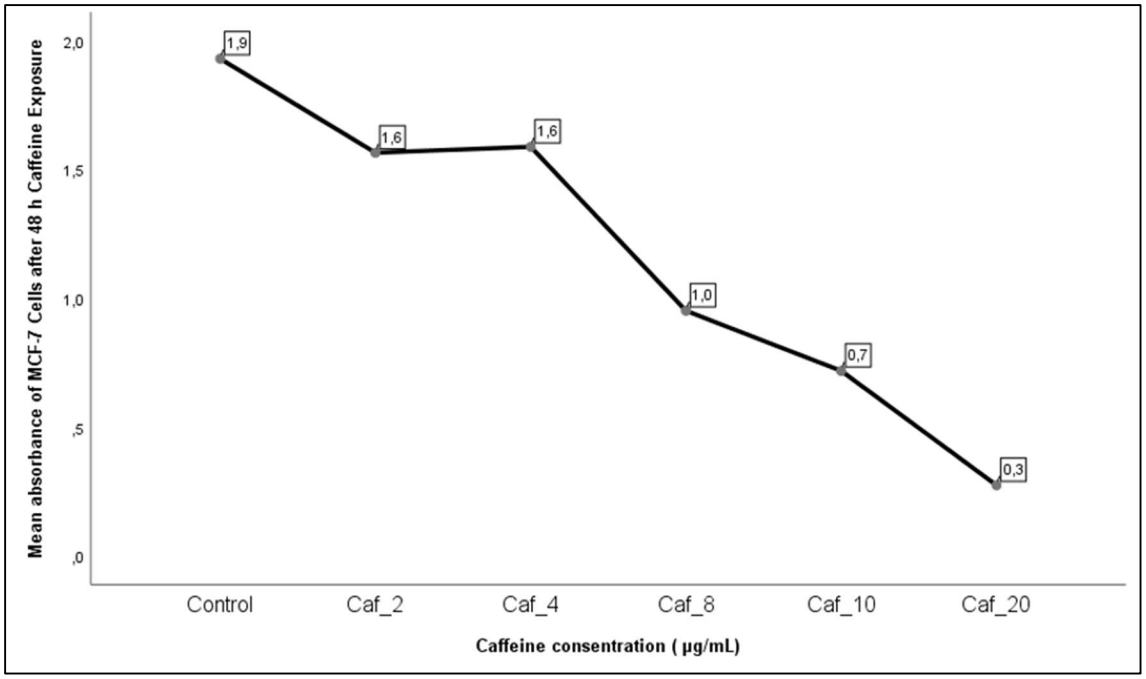
Graph 1: Mean absorbance of MCF-7 Cells after 24h of exposure



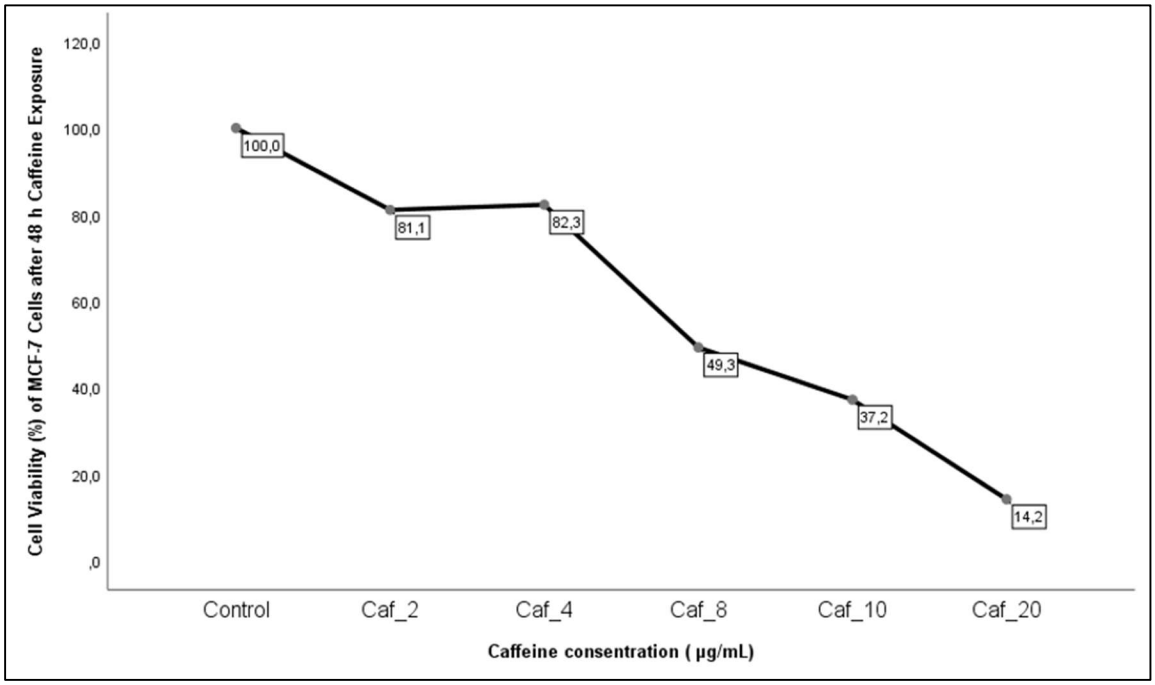
Graph 2: Cell Viability of MCF-7 Cells after 24h exposure



Graph 3: Mean absorbance of MCF-7 Cells after 48h of Exposure



Graph 4: Cell Viability of MCF-7 Cells after 48 h of Exposure



All graphs have demonstrated a proportional/gradual decrease in general in both MCF-7 Cell viability and percentage absorbance levels in the hour spans of 24-48 h whereas, at the higher concentrations starting from 4mg/mL, the graph illustrated a rapid slump instead of following a fractional decrease.These results strongly support my hypothesis which stated that the lower and moderate concentrations of caffeine (2, 4,mg/mL mg/mL) would have minimal to no effect on cellular viability, whereas higher concentrations of caffeine (≥8 mg/mL) would have a significant effect in decreasing the cell viability via oxidative stress.

Conclusion

Our findings are consistent with literature showing that the effects of caffeine vary depending on concentration and duration of exposure. Various in vitro studies have reported that repair and antioxidant responses can be enhanced at relatively low concentrations, whereas apoptosis/necrosis becomes more pronounced at higher concentrations and/or longer exposure times. Since the lowest dose in our design was 2 mM, we did not systematically evaluate the possible "protective window" in the very low dose ranges; therefore, we do not draw conclusions for these ranges. On the other hand, we quantitatively demonstrated that proliferation was suppressed in a dose-/time-dependent manner in the 2–20 mM and 24–48 hour range, and the effect became stronger towards the 48th hour [2, 6, 7].

The findings that caffeine can down-regulate ERα levels and ER-dependent signaling in ER-positive models are conceptually consistent with the antiproliferative pattern we detected in MCF-7. Previous studies have indicated that caffeine (and/or coffee components) can inhibit the cell cycle by attenuating the ERα/cyclin D1 axis and associated growth pathways, which in some contexts can potentiate the effect of antiestrogenic therapies. Our unique contribution is that we quantitatively defined the threshold and window of effect in MCF-7 under the caffeine-only condition—without the use of any concomitant agents (tamoxifen, extract, etc.): a persistent suppression that becomes evident at ≥8–10 mM, peaks at 20 mM, and extends to 48 h. We believe that this detailed dose-time profile will generate rational hypotheses for future confirmatory measurements and possible combination designs with markers such as ERα/cyclin D1/pAkt [3, 4, 8, 9].

Other cancer/cell models that demonstrate that caffeine's effects are context-specific complement this picture: increased tumor suppression with some chemotherapies has been

reported in liver cancer (HCC) models; enhanced light/drug response in glioblastoma; and sensitizing or relatively protective effects have been reported in some colon cancer settings. This variability explains why literature results are not always in the same direction, depending on different cancer types, cellular microenvironment, concomitant treatments and dose-duration parameters [10,11] .

The discrepancy between studies reporting “no effect/protective” findings of caffeine at low doses and significant cytotoxicity at medium-high doses appears to be due to methodological factors such as concentration range (µM↔mM), exposure time, solvent/vehicle differences, serum availability, and sensitivity of the endpoint tests used, as well as cell line-specific sensitivities. Our 2–20 mM, 24–48 h design clarifies the pressor effect of caffeine at mid-range doses and prolonged durations in MCF-7, while retaining a deliberate limitation of not commenting on very low dose windows.

Evaluation:

Although, the effects of caffeine on cancer biology have been examined since 1970s, the differentiation of responses dependent to dosage and timing related to tumor subtypes remained inconclusive [1] . In this study I evaluated the effect of caffeine focusing on breast cancer cell proliferation in the MCF-7 cell line, which represented the estrogen receptor positive (ER+) subtype, at the dosage range of 2-20 mM and 24-48 h of exposure times. Our outcomes have demonstrated that caffeine exhibits a significant and dosage-dependent antiproliferative/cytotoxic effect in vitro (Mechanistic considerations in chemotherapeutic activity of; Oxidative Stress in Caffeine Action on the Proliferation and Death of Human Beings) [2,3] . It was also illustrated that the suppression became evident at ≥8-10 mM, reached the highest level at 20mM, and the effect continued until 48 hours. These results are seen to be consistent with caffeine modulating the cell cycle and inducing apoptosis. While human data mostly demonstrates a neutral picture in terms of breast cancer risk in the general human population, different results have been stated varying in subgroups based on factors such as menopausal status, body mass index (BMI), and genetic predisposition [4,5] . Therefore, it should be taken into account that the clinical implications of the antiproliferative affects I have shown at the experimental level may be delicate to different host factors.

The major component of the antitumor response observed by caffeine in MCF-7 cells is oxidative stress triggered by intracellular reactive oxygen species (ROS) accumulation and accompanying mitochondrial dysfunction. While moderate to high concentrations of caffeine increase lipid peroxidation and DNA damage markers, the increase in the cell's antioxidant defenses cannot fully reverse the damage and the process often ends with apoptosis. The fact that viability did not fully recover even when specific scavengers that clean certain ROS

species were added suggests that cytotoxicity cannot be explained by a single oxidant species; rather, it is the combined effect of multiple ROS species [2,3] .

Limitations and Further Investigations

This study has several limitations. First, our study was conducted in a single cell line (MCF-7, ER-positive) and under in vitro conditions; therefore, the results may not fully reflect the effects of metabolism, tumor microenvironment, and immunity in the body. We also measured only proliferation/viability; ERα level, cell cycle phases, apoptosis–necrosis distinction, mitochondrial membrane potential, and ROS subtypes were not directly measured. We cannot comment on the "low-dose protective window" as our dose range (2–20 mM) does not cover very low concentrations; the time range (24–48 h) also suggests limited early/late effects. For generalizability of the findings, it is recommended to repeat them in HER2-positive and triple-negative lines, preferably in 3D culture/spheroid and appropriate in-vivo models; also to test ranges closer to physiological doses and rational combinations.

Caffeine alone showed significant dose- and time-dependent antiproliferative effects in MCF-7 cells at 2–20 μM and 24–48 h exposure. This effect most likely occurs through ROS-mediated oxidative stress and mitochondrial damage, along with attenuation of ERα signaling and activation of the p53 pathway. Our findings are consistent with mechanisms reported in the literature; however, due to differences in methodology/physiology between studies, mechanistic confirmations and clinically relevant dose/combination studies are required [2, 3, 8].

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Appendix

T.C.
SAĞLIK BİLİMLERİ ÜNİVERSİTESİ
Gülhane Tıp Fakültesi Dekanlığı
Fizyoloji Anabilim Dalı Başkanlığı

To whom it may concern,

This is to certify that [redacted] has done her IB diploma programme "How do varying concentrations of pure caffeine affect the viability of MCF-7 breast cancer cells?" titled essay experiments at the Physiology Department of Gülhane Medical Faculty by herself with the supervision of Asst. Prof. Dr. Zehra Çiçek. 03.03.2026

Sincerely,

Prof. Dr. Mehmet Özler
University of Health Sciences,
Gülhane Faculty of Medicine
Department of Physiology, Ankara, Türkiye