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Evaluation of the Antiproliferative Effects of Epigallocatechin Gallate (Green Tea) in MDA-MB-231 Human Breast Cancer Cells: An *In vitro* Study

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Abstract **Original Research Article**

Background: Epigallocatechin gallate (EGCG), a catechin present in green tea, has been investigated for effects on cancer-cell growth. This study examined changes in metabolic activity and apoptosis-associated staining in the oestrogen-receptor-negative MDA-MB-231 human breast cancer cell line following EGCG exposure. ***Methods:*** MDA-MB-231 cells were cultured and exposed to five nominal EGCG treatment amounts described in the dissertation as 10, 50, 100, 150 and 200 µg, alongside untreated controls. Cellular MTT reduction was assessed at 24 and 48 hours, and a Guava Nexin assay was analysed by flow cytometry. The original report describes means ± standard error of the mean from six observations and paired t tests but does not establish the number of independent biological replicates. ***Results:*** The MTT assay demonstrated reduced absorbance in EGCG-treated MDA-MB-231 cells compared with controls, with the strongest inhibition reported at higher treatment amounts. Microscopy documented changes in cell appearance after EGCG exposure. Flow cytometry results were evaluated at 24 and 48 hours, with the original statistical tables presented in the Results section. ***Conclusions:*** EGCG treatment was associated with inhibition of MDA-MB-231 cell growth in the experimental conditions studied. The MTT, microscopy and flow-cytometric observations are presented together to characterise the cellular response to EGCG.

Keywords: EGCG; epigallocatechin gallate; MDA-MB-231; breast cancer; MTT; flow cytometry.

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1. INTRODUCTION

Breast cancer is a heterogeneous disease, and experimental cell models provide a means of investigating candidate compounds under controlled conditions. MDA-MB-231 is an oestrogen-receptor-negative human breast cancer cell line commonly used to study aggressive breast cancer phenotypes. Its use in vitro does not, however, reproduce the tumour microenvironment, drug exposure or clinical outcomes of patients.

Green tea contains catechins, including epigallocatechin gallate (EGCG) (Graham, 1992), which has attracted interest in its interactions with cellular pathways involved in proliferation and survival. Earlier experimental reports described cell-cycle and apoptosis-associated changes after EGCG treatment in carcinoma cells (Ahmad *et al.,* 1997), and effects on HGF/Met signalling in breast epithelial models (Bigelow and Cardelli, 2006). The original dissertation also cites work on EGCG in MDA-MB-231 cells, meaning that inhibition of this cell line by EGCG cannot be presented as a newly discovered phenomenon.

MTT assays were used to assess the response of MDA-MB-231 cultures to EGCG treatment, alongside microscopic observations and flow-cytometric assessment.

This study investigated the effects of EGCG on MDA-MB-231 cells following 24 and 48 hours of exposure, using MTT assays, microscopy and flow cytometry.

2. MATERIALS AND METHODS

2.1 Cell line and culture conditions

MDA-MB-231 human breast cancer cells were reported to have been obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium supplemented

with 5% fetal bovine serum, L-glutamate, penicillin and streptomycin at 37 °C in humidified 5% CO_2. Cultures were maintained at approximately 60–70% confluence in T-75 flasks. Cells were detached using 0.25% trypsin and 0.02% EDTA and counted by trypan blue exclusion using a haemocytometer.

2.2 Seeding optimisation and exposure

Seeding densities between 50,000 (Fig 2.2.1) and 250,000 cells/mL (Fig 2.2.2) were evaluated in six- and 24-well plates. A nominal density of 50,000 cells/mL was selected on the basis of microscopic appearance and confluence over time. After an overnight attachment period, cells were exposed to EGCG supplied by Sigma-Aldrich. A cell-and-medium control was included alongside the EGCG treatment groups.

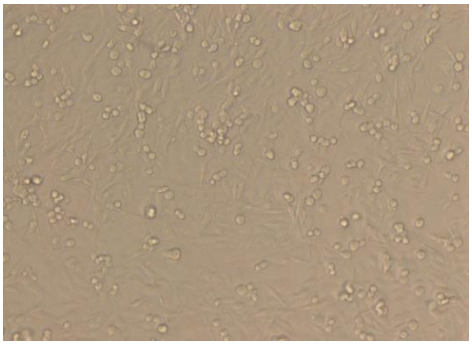


Fig 2.2.1

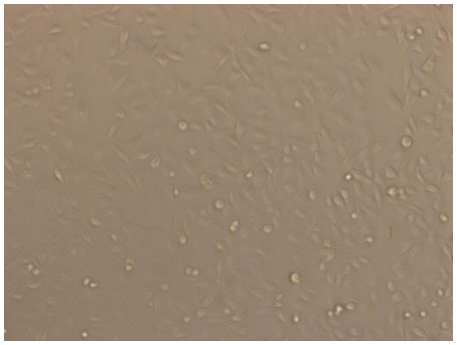


Fig 2.2.2

2.3 MTT assay

MTT reagent was prepared at 5 mg/mL in phosphate-buffered saline and protected from light. There was removal of 300 µL of medium, addition of 50 µL of MTT solution, and incubation for three hours at 37 °C. The supernatant was then removed, wells were rinsed with 500 µL phosphate-buffered saline, and 300 µL dimethyl sulfoxide was added. After mixing and 15 minutes of orbital shaking at room temperature, absorbance was read at 590 and 620 nm on a FLUO star Omega plate reader. Absorbance was recorded following MTT treatment and dissolution of the resulting formazan product.

2.4 Guava Nexin flow-cytometric assay

For apoptosis-associated staining, culture supernatant and phosphate-buffered-saline washes were collected from six-well plates at 24 and 48 hours. Adherent cells were detached with 200 µL trypsin-EDTA for approximately three minutes at 37 °C, combined with medium, and centrifuged for five minutes at 1,000 rpm. Pellets were resuspended in 175 µL complete medium and stained with 25 µL Guava Nexin reagent. Samples were protected from light and incubated for 20 minutes at room temperature with shaking at 200 rpm, then analysed on a Guava easyCyte flow cytometer.

Flow-cytometric analysis was performed using the Guava Nexin assay to assess apoptosis-associated changes in the treated cell populations.

2.5 Statistical analysis

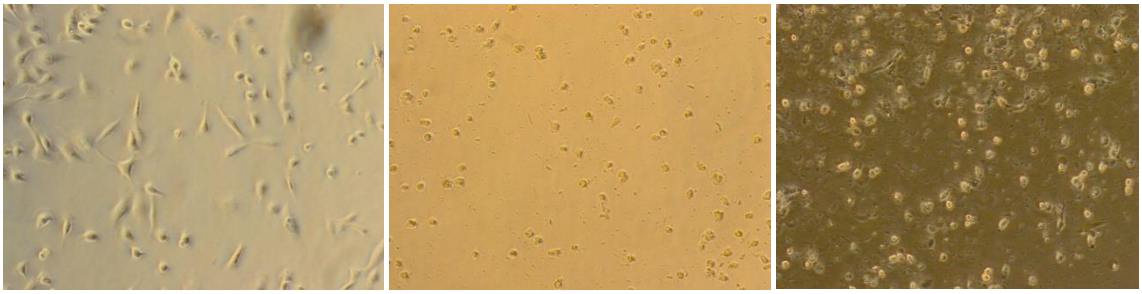
Results were expressed as mean ± SEM of six observations. Data were analysed by using paired-sample T-test. Significance threshold of $p < 0.05$ was defined.

3. RESULTS

3.1 Culture establishment and qualitative morphology

The seeding-optimisation micrographs show MDA-MB-231 cultures after 24, 48 and 96 hours at a selected nominal density of 50,000 cells/mL. Images were also recorded after 24 and 48 hours of exposure, including control, 10-µg and 200-µg nominal treatment conditions.

EGCG treated cells showed increased apoptosis (Fig. 3.1.1 B,C) and decreased proliferation (Fig 3.1.1 D-F) when compared with the untreated control (Fig. 3.1.1 A). A decrease in EGCG treated cells indicated the anti-proliferative effect of these polyphenols.



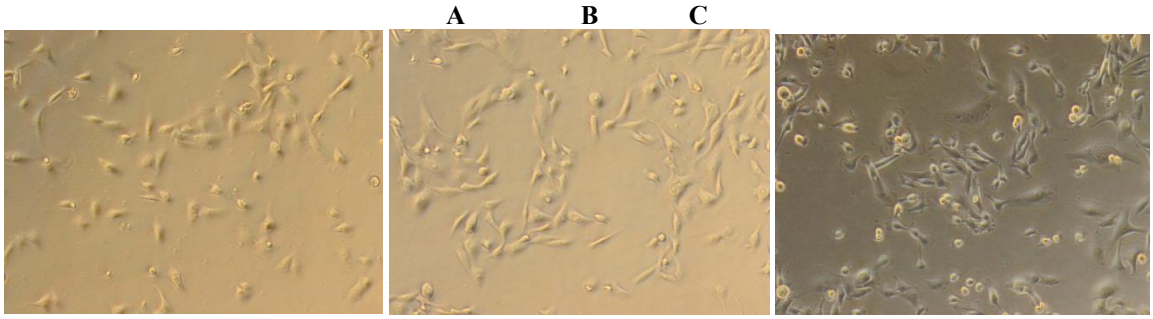


Fig. 3.1.1 (A) Control, (B) 200ug EGCG treated, (C) 150ug EGCG treated, (D) 100ug EGCG treated, (E) 50ug EGCG treated (F) 10ug EGCG treated

3.2 MTT signal after EGCG exposure

Figure 3.2.1 presents MTT measurements after 24 and 48 hours of EGCG exposure across the control and treatment groups. The results show a concentration-associated reduction in the MTT signal, as described in the original study.

After examining the effect of increasing concentration of EGCG on the growth of oestrogen receptor negative MDA MB 231 human breast cancer cells for 24 and 48 hours using MTT assay, it was seen that treatments with EGCG (10µg-200µg) inhibited the growth of MDA MB 231 breast cancer cells in a concentration dependent manner with an IC50 of 100µg for EGCG at 24 and 48 h. (Fig. 3.2.1) extensive inhibition of cell growth was observed in polyphenol treatment groups receiving high concentrations (>100µg/ml) of EGCG.

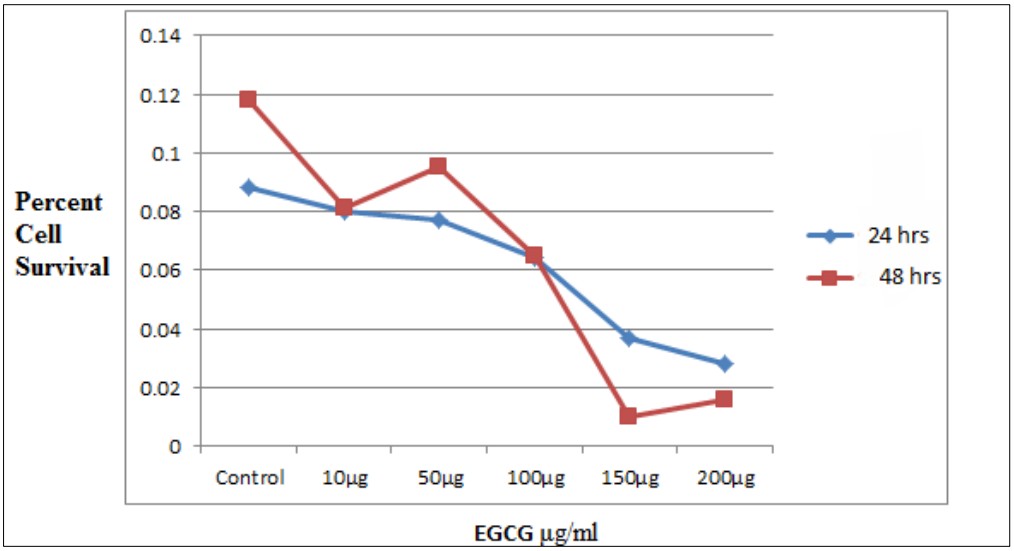


Fig. 3.2.1. Extensive inhibition of cell growth at 24 h and 48 h. Mean values of MTT Assay.

MTT assay values were statistically analysed by using paired sample t-test (Fig. 3.2.2 & 3.2.3) and control was compared with all other different concentrations and it was seen that p-value was less than 0.05 and thus the hypothesis was rejected and mean values obtained show a significant result.

MTT assay at 24 HOURS

Fig. 3.2.2 The sig value is less than 0.05 and thereby the resulting hypothesis. i.e since the values are less than 0.05 the hypothesis is rejected and the mean values obtained show a significant variation

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Concentration	3.5000	6	1.87083	.76376
	Absorbance	.06233	6	.024533	.010016

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Concentration & Absorbance	6	.292	.575

Paired Samples Test									
		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Concentration - Absorbance	3.437667	1.863814	.760899	1.481714	5.393619	4.518	5	.006

MTT assay at 48 HOURS

Fig. 3.2.3 - The sig value is less than 0.05 and thereby the resulting hypothesis. i.e since the values are less than 0.05 the hypothesis is rejected and the mean values obtained show a significant variation

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Concentration	3.5000	6	1.87083	.76376
	Absorbance	.06417	6	.043329	.017689

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	Concentration & Absorbance	6	.132	.803

Paired Samples Test									
		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Concentration - Absorbance	3.435833	1.865604	.761630	1.478002	5.393664	4.511	5	.006

3.3 Flow-cytometric apoptosis-associated findings

Flow cytometric analysis was conducted after 24 and 48 hours of EGCG exposure. The original statistical results for both time points are presented below.

Apoptosis, or programmed cell death, is an important and active regulatory pathway of cell growth and proliferation. Cells respond to specific apoptotic induction signals by initiating intracellular processes that result in characteristic physiological changes. Among these changes are externalization of phosphatidylserine to the cell surface, depolarization of mitochondrial membranes, cleavage and degradation of specific cellular proteins, compaction and fragmentation of nuclear chromatin, loss of cell membrane integrity, and cellular shrinkage. Apoptosis is a kinetic event, and the time during which cells exhibit the "markers" of apoptosis is variable and short. The manifestations of apoptotic progression are affected by several factors: 1) the cell type, 2) cell condition, 3) the treatment to induce apoptosis, 4) the method used to characterize the physiological change.

Statistical analysis done for Guava Easycyte flow cytometer for 24 and 48 hours (Fig. 3.3.1 & 3.3.2) shows p-value was greater than 0.05 and thus the hypothesis was accepted and mean values obtained do not show a significant result.

FOR 24 HOURS

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Concentration	3.5000	24	1.74456	.35611
	Observance	25.2679	24	35.88790	7.32559
Pair 2	Condition	2.5000	24	1.14208	.23313
	Observance	25.2679	24	35.88790	7.32559

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Concentration & Observance	24	.015	.943
Pair 2	Condition & Observance	24	.246	.246

Fig. 3.3.1 P value is greater than 0.05 thus accepting the hypothesis.

Paired Samples Test									
		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Concentration - Observance	-21.76792	35.90339	7.32875	-36.92859	-6.60725	-2.970	23	.007
Pair 2	Condition - Observance	-22.76792	35.62381	7.27168	-37.81053	-7.72530	-3.131	23	.005

FOR 48 HOURS

Fig. 3.3.2 P value is greater than 0.05 thus accepting the hypothesis. Sig. shows non-significant result.

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Concentration	3.5000	24	1.74456	.35611
	Observance	25.0750	24	36.53036	7.45673
Pair 2	Condition	2.5000	24	1.14208	.23313
	Observance	25.0750	24	36.53036	7.45673

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	Concentration & Observance	24	-.003	.989
Pair 2	Condition & Observance	24	.291	.167

Paired Samples Test									
		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Concentration - Observance	-21.57500	36.57734	7.46632	-37.02026	-6.12974	-2.890	23	.008
Pair 2	Condition - Observance	-22.57500	36.21418	7.39219	-37.86691	-7.28309	-3.054	23	.006

4. DISCUSSION

EGCG treatment was associated with a decrease in the MTT assay signal in MDA-MB-231 cultures after 24 and 48 hours. The greatest inhibition was observed at the higher treatment amounts, consistent with the microscopic observations of altered cell growth.

The use of multiple treatment amounts and two time points enabled the study to examine EGCG-associated changes in cell growth over a range of experimental conditions. Flow cytometry was used as an additional method to assess apoptosis associated cellular changes.

The MTT findings suggest that EGCG has antiproliferative activity in this in-vitro model. Similar effects of green tea catechins on tumour-cell proliferation and cellular signalling have been described in previous experimental studies.

The flow-cytometric results were assessed at 24 and 48 hours. The statistical comparisons in the original study reported p values greater than 0.05 for the analysed flow-cytometry measurements.

EGCG has been investigated in connection with signalling pathways involved in cell proliferation, apoptosis and tumour progression. The observations from the present study contribute additional experimental findings in the MDA-MB-231 cell model.

The results are consistent with previous research examining EGCG activity in breast cancer cell models and support continued investigation of green tea polyphenols in experimental cancer biology.

Taken together, the MTT assay, microscopy and flow-cytometry findings provide a description of the

cellular response to EGCG exposure under the conditions used in this study.

5. CONCLUSION

EGCG exposure was associated with reduced MTT assay measurements and altered cellular morphology in MDA-MB-231 breast cancer cells at 24 and 48 hours. These observations support an antiproliferative effect of EGCG in this in-vitro model.

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