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Effect of caffeine versus hydrogen peroxide on human skin fibroblast cell line cytotoxicity, cell cycle phases, and apoptosis

Sara Adel Taha Denewer¹, Heba Elsabaa^{2,3}

Aim: The study reported the effect of caffeine and H₂O₂, concerning cytotoxicity followed by cell cycle analysis and programmed cell death against the human skin fibroblast cell line (HSF).

Materials and Methods: HSF treated by 1.6 mM concentration sub-lethal dose of H₂O₂ and caffeine with various concentrations. A flow cytometry test was accomplished to estimate the effect of caffeine and H₂O₂ on the HSF cell line.

Results: This study postulated that caffeine had a lower cytotoxicity effect on the HSF cell line with IC₅₀>5 μM in contrast to H₂O₂ showed cytotoxicity above 1.6 μM. As compared to the control cells, caffeine-treated cells showed a significant decline in the early and late apoptotic cell content by -60% and -48%, respectively. However, H₂O₂-treated cells exhibited significant elevations in the early apoptotic content as compared to the control cells.

Conclusions: The study showed that H₂O₂ had an effect of cytotoxicity on HSF and arrested more cells in the S phase in comparison to caffeine. Caffeine is safer on the HSF cell line.

Key words: HSF, H₂O₂, Caffeine, Cytotoxicity, Flow cytometry.

1. Lecturer at Badr University Cairo (BUC), Egypt.
2. Professor of Oral Biology, Faculty of Dentistry, Mansoura University, Egypt
3. Chair of Oral Biology & Pathology Department Faculty of Oral & Dental Medicine, Badr University in Cairo BUC, Egypt.
Corresponding author: Sara Adel Taha Denewer, email: sasoodenewer@gmail.com

Introduction

Fibroblasts have a crucial role in preserving the function and structure of the dermis, in addition to wound healing. Consequently, fibroblasts show a very significant part in the procedure of aging of the skin, and they epitomize a potential satisfying target to avoid this progression.^{1,2} Skin is more liable to the factors of the environment because it is in continuous contact with the surrounding environment. Damage caused by oxidative stress results in early cell aging and encourages the production of melanin and inflammation in the skin.³

Concurrently, H₂O₂ is considered a kind of reactive oxygen species (ROS) that cross the cell membrane simply to encourage the production of free radicals and lipid peroxidation, which hinder proliferation of the cell and encourage the cell death. H₂O₂ is extensively used in vitro studies to release oxidative stress. Ultraviolet (UV) radiation produces H₂O₂ and other ROS that may cause impairment to DNA, RNA, lipids, and proteins in addition to cell death in the tissues subjected to the ultraviolet, such as skin.⁴

Caffeine is considered an alkaloid plant found in many soft drinks, coffee, tea, Coca-Cola, and many chocolates frequently used around the world.⁵ It is taken up from the gastrointestinal tract (GIT) and is noticeable in all fluids of the human body.⁶ It has been described as a scavenger for free radicals, as they possess many antioxidant activities in vitro and in vivo.^{7,8} Hence, the aim of the present work was to evaluate the possible effects of caffeine and H₂O₂ on the skin fibroblasts.

Material and methods

A) Cell line culture:

The Human Skin Fibroblasts were purchased from Nawah Scientific™ (Mokatam, Cairo, Egypt). The cells were kept in basal medium of Dulbecco's Modified

Eagle Medium (DMEM) accompanied with 100 mg/mL streptomycin, 100 units/mL penicillin, and 10% of inactivated heat fetal bovine serum in a humidified, 5% (v/v) CO₂ at 37 °C atmosphere.

B) Study design:

1. Control group: The HSF cells were not subjected to any test substance (Caffeine or H₂O₂). **2. Hydrogen peroxide (H₂O₂) group:**

HSF treated by 1.6 mM concentration sub-lethal dose of H₂O₂ with various concentrations (0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, 1mM, and 1.6 mM) for 1 h.

3. Caffeine group: HSF cells treated with various concentrations of caffeine 100 mg vail (0.01 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM, and 5 mM) for 48 hours.

The mean values of the caffeine and H₂O₂ groups were considered by calculating the mean values of all measures of the different concentrations.

C) Power analysis and sample size:

The test goals to compare 2 population means, with the null hypothesis that the means are equal and the alternative hypothesis that they are different.

The calculations are achieved presumptuous a significance level (α) of 0.05 and a standard deviation of 0.05. The text presents result for an exact scenario where the difference between the two means is 0.24, and the target power is set to 0.8 (80%).⁹

The required sample size for each group is 3, which results in an actual power of 0.988796 (approximately 98.88%).

Power and Size of Sample

Two-Sample t Test

Testing mean 1 = mean 2 (versus \neq)

Calculating power for mean 1 = mean 2 + difference

$\alpha = 0.05$ Expected standard deviation = 0.05

The sample size of the study was 3 dishes from cell line culture to follow allocation ratio 1:1, randomization and blinding. The study design was approved by ethical committee BUC-IACUC-231015-45

D) Investigations of the parameters:

1. Cytotoxicity test:

Evaluating cell viability by Sulforhodamide B (SRB) assay. After drug exposure for 48-hour, fixing cells by replacing media with 150 μ L of 10% Trichloroacetic acid (TCA) and laid at 4 °C for one hour. The cells were cleaned using distilled water (5 intervals) after eliminating TCA solution. Adding 70 μ L aliquots of solution of SRB (0.4% w/v) and incubating at room temperature for 10 minutes in an unlighted place. After that, dishes were cleaned 3 intervals with 1% concentration of acetic acid and left the whole night to dry. After then, adding 150 μ L of TRIS (10 mM) to melt protein-bound SRB stain; measuring the absorbance at 540 nm utilizing a microplate reader BMG LABTECH®- FLUOstar Omega (Ortenberg, Germany).¹⁰

2. Cell Cycle Distribution:

Trypsinization used to collect cells and cleaned two times with Phosphate Buffered Saline (PBS) ice-cold at 7.4 pH, after treatment with test compounds for a day/2 days and paclitaxel (1 μ M) as a positive control for 24h. Resuspension of cells was done in 2 milliliters of 60 percent of ethanol ice-cold, then, implanted for a day at 4°C for fixation. The fixed cells were immersed two times with PBS after that resuspended in PBS (1 mL) carrying 10 μ g/mL propidium iodide (PI) with RNase A 50 μ g/mL. Placement in an unlighted room at 37° C temperature after 20 minutes, examining cells for DNA by applying flow cytometry test via FL2 (λ_{ex}/em 535/617 nm) signal detector (ACEA Novocyte™ flow cytometer, ACEA Inc.,

CA, San Diego, USA). For each model, 12,000 trials were obtained. Distribution of the cycle of the cell was determined by means of software ACEA NovoExpress™ (ACEA Biosciences In Company, San Diego, USA).¹¹⁻¹⁴

3. Flow cytometry assay:

Studying of all aspects of apoptosis from induction through receptors of the surface, to fragmentation of DNA (late stages) happened by flow cytometry. Annexin V-FITC Apoptosis Staining / Detection Kit is utilized in 10 minutes, one-step staining method to notice apoptosis through staining molecules of phosphatidylserine which have transferred to the outside of the cell membrane. The kit can distinguish apoptosis versus necrosis when conducting both Annexin FITC-V and Propidium Iodide (PI) staining.^{11, 12} The flow chart of the study methodology is illustrated in figure (1).

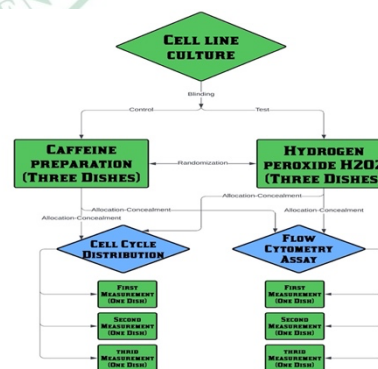


Fig. 1: Flow chart of the study methodology

E) Statistical analysis

Statistics were investigated by utilizing version 23 software of statistical software package (IBM-SPSS). Kolmogorov–Smirnov test revealed that the raw statistics normally were dispersed. One-way ANOVA was indicated to study the outcome of regimen on the studied parameters. To explain the statistical differences among the studied groups, the least significant difference (LSD) test was

indicated. Data were showed as mean ± standard error of mean.

Results

1. Cytotoxicity test results:

Dose curve response:

As shown in figures 2 & 3 Caffeine showed cytotoxicity (IC50) at a concentration above 5 μM, while H2O2 revealed cytotoxicity (IC50) at a concentration above 1.6 μM. On the other side H2O2 showed a sublethal effect on HSF at a concentration below 1.6 μM for 1 hour compared to caffeine.

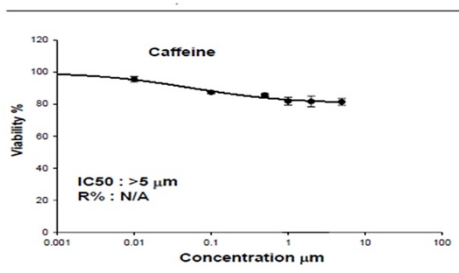


Fig 2. comparing cytotoxicity among different caffeine concentrations.

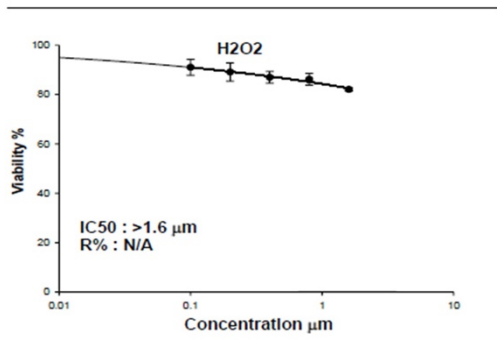


Fig 3. comparing cytotoxicity among different caffeine and H2O2 concentrations.

2. Test of flow cytometry:

A. Effect on apoptotic content:

The content of early and late apoptotic cells after regimen with caffeine and H2O2 was reported (Table 1 and Fig 4).

As compared to the control cells, caffeine-treated cells revealed a significant decline in the early (p=0.022) and late (p=0.000) apoptotic cell content by -60% and -48%, respectively. However, H2O2-treated cells

exhibited significant (p=0.000) elevations in the early apoptotic content as compared to the control cells (by +141%) as well as to the Caffeine-treated cells (by +501%).

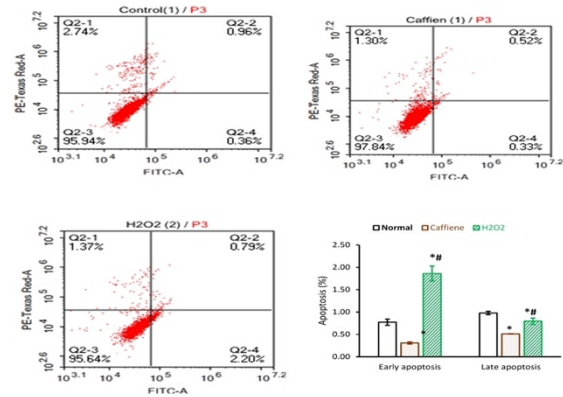


Fig 4. A representative flow cytometry-based apoptosis in the control, caffeine and H2O2-treated cells. Quantitative analysis of early and late apoptosis. Data were displayed as mean ± standard error. *: represents a significant difference (p<0.05), as compared to the control group. #: represents a significant difference (p<0.000), as compared to the caffeine groups.

Table 1. The early and late apoptosis percent in all the control, caffeine, and H2O2-treated cells. Data were displayed as mean ± standard error.

	Control	Caffeine	H2O2
Early apoptosis	0.77 ± 0.07	0.31 ± 0.03*	1.86 ± 0.17##
Δ1, p1	---	-60%, 0.022	+141%, 0.000
Δ2, p2	---	---	+501%, 0.000
Late apoptosis	0.98 ± 0.04	0.51 ± 0.001*	0.80 ± 0.07##
Δ1, p1	---	-48%, 0.000	-19%, 0.000
Δ2, p2	---	---	+55%, 0.000

#: shows different of significance (p1<0.05), in comparison to the control group, #: shows different of significance (p2<0.05), in comparison to the caffeine groups. Δ1, Δ2: percent of change, as compared to the control cells and caffeine-treated cells, respectively.

A. Effect on cell cycle

The changes in cell cycle arrest after treatment with caffeine and H2O2 were presented (Table 2 and Fig 5). In all cell groups, the highest and smallest percentages of cell cycle arrest were detected at G0/G1 and Sub-G1 phases, respectively. However, no significant differences were detected at G0/G1 phase, among all the studied groups. As compared to the control cells, H2O2-treated cells revealed a significant (p=0.001) elevation in cell cycle arrest percent at S-phase by +27%. As compared to caffeine-treated cells, a significant (p=0.004)

elevation in the percent of cell cycle arrest was noticed, at S-phase by +18%, after treatment by H₂O₂.

At the G₂/M phase, a significant ($p=0.005$) reduction in the cell cycle arrest percent was detected in the H₂O₂-treated cells by -7%, as compared to both the control and caffeine-treated cells.

In sub G₁ phase, the caffeine-treated cells showed significantly higher percent of cell cycle arrest than the control (by +25%). However, H₂O₂-treated cells showed a marked ($p=0.002$) decline in the percent of cell arrest at sub G₁, by -29%, as compared to the caffeine-treated cells.

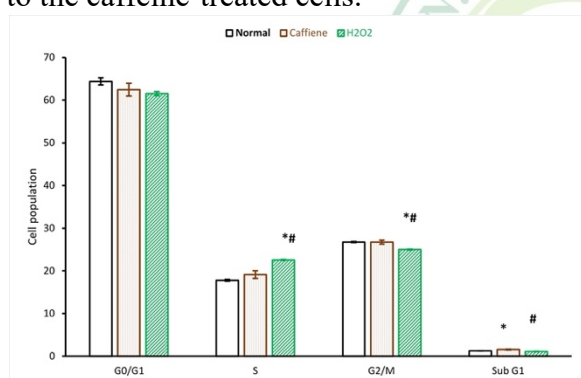


Fig 5. Caffeine and H₂O₂-induced cell arrest in cell cycle. Data were displayed as mean \pm standard error. *: represents a significant difference ($p<0.05$), as compared to the control group. #: represents a significant difference ($p<0.05$), as compared to the caffeine groups.

Table 2. Caffeine and H₂O₂-induced cell arrest in cell cycle. Data were displayed as mean \pm standard error.

Phase	Control	Caffeine	H ₂ O ₂
G0/G1	64.39 \pm 0.83	62.46 \pm 1.50	61.51 \pm 0.50
$\Delta 1, p1$	---	-3.0%, 0.235	-4.0%, 0.096
$\Delta 2, p2$	---	---	-2%, 0.537
S	17.80 \pm 0.19	19.14 \pm 0.88	22.56 \pm 0.11*#
$\Delta 1, p1$	---	+7.0%, 0.123	+27%, 0.001
$\Delta 2, p2$	---	---	+18%, 0.004
G2/M	26.76 \pm 0.13	26.75 \pm 0.47	24.98 \pm 0.12*#
$\Delta 1, p1$	---	0.0%, 0.975	-7.0%, 0.005
$\Delta 2, p2$	---	---	-7.0%, 0.005
Sub G1	1.27 \pm 0.02	1.58 \pm 0.09*	1.13 \pm 0.05#
$\Delta 1, p1$	---	+25%, 0.011	-11%, 0.150
$\Delta 2, p2$	---	---	-29%, 0.002

*: shows different of significance ($p1<0.05$), in comparison to the control group, #: shows different of significance ($p2<0.05$), in comparison to the caffeine groups. $\Delta 1, \Delta 2$: percent of change, as compared to the control cells and cells treated with caffeine, respectively.

Discussion

The human cells are affected by many factors. One of these factors is the reactive oxygen species. Abelmeguid et al.¹⁵ reported that a rise in ROS generation may result in oxidative stress, a decline in antioxidant systems, or both. This damage to proteins, DNA, and organelles of cell finally lead to cellular death.

The current study assessed the cytotoxicity effect of H₂O₂ in comparison to caffeine on HSF. Our results indicated that caffeine had a safe effect at different concentrations on HSF after 48 hours and to cause cytotoxicity above 5 μ M. While H₂O₂ shows more toxic effects on HSF. The concentration of H₂O₂ above 1.6 μ M can cause 50% death of cells for 1-hour exposure. The inducing effect of H₂O₂ to apoptosis on HSF was found to be in accordance with Gloria postulated that treatment of human gingival fibroblasts with H₂O₂ produced cytotoxic effects and encouraged apoptosis.¹⁶ In accordance to this study Shi et al.¹⁷ showed that stimulation of H₂O₂ at a concentration of 100 μ mol·L⁻¹ for two hours was selected as the demonstrating condition regarding to cell cytotoxicity, and 50% was the rate of survival. The count of cells of HSF was reduced, and the shape of cell altered from normal thin cell to round short to death.

Caffeine is the most recurrently consumed neuroactive medicine in the whole world. Caffeine has been described to affect function of cell cycle, encourage programmed cell death or apoptosis and disturb regulatory proteins for cell cycle. Though the properties of caffeine have been deeply examined, much of the study data concerning effects of caffeine on the cell cycle and proliferation look like to be indistinct.¹⁸

The present study reported that caffeine has low cytotoxicity and less apoptotic effect in comparison to H₂O₂. In accordance with Silverberg et al.¹⁹ who

reported that HSF cell line were treated with caffeine after being endangered by H₂O₂-fortified necrosis, which led to numbers of cells increased and enhanced morphology of the cell, the protective mechanism of which looked to be facilitated by a mechanism besides the function of antioxidant.

Stimulatingly, other studies have also postulated diverse effects, reporting that caffeine can increase proliferation of cell maybe representing cell-type-specific effects.^{19, 20}

Another study referred to caffeine limits proliferation of cell and postponements migration in a dose-dependent manner in human skin ex vivo and keratinocytes in vitro.²¹

Conclusions

Caffeine is safer on human skin fibroblast in comparison to H₂O₂ which showed early apoptosis and arrested more cell in S phase and less population of cells entered in M phase in comparison to control and caffeine groups.

Ethical statement

All stages of this study were revised and accepted by the BUC-Institutional Ethical Committee at Badr University in Cairo, number of approvals: (BUC-IACUC-231015-45).

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Declaration of Competing Interest

The authors state that no known challenging financial benefits or personal relationships that would have seemed to affect the work described in this study.

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