

Original Article

EVALUATING THE LEVELS OF OXIDATIVE DNA DAMAGE IN HUMAN LYMPHOCYTES IN RESPONSE TO CAFFEINE USING COMET ASSAY (SINGLE CELL GEL ELECTROPHORESIS)

ESTABRAQ A. AL-WASITI¹, SAHAR ABDAL WHAB AL-SHABAN², ANAM R. AL-SALIHI¹, HAYDER A. AL-AUBAIDY³

¹College of Medicine, Al-Nahrain University, Baghdad, Iraq, ²College of Medicine, Al-Mustansiriya University, Baghdad, Iraq, ³School of Medicine, University of Tasmania, Hobart, TAS, Australia
Email: h.alaubaidy@utas.edu.au

Received: 09 Feb 2016 Revised and Accepted: 20 Apr 2016

ABSTRACT

Objectives: Caffeine (coffee, cola, and tea) is the most widely consumed beverages worldwide. The current study aims to evaluate the effects of caffeine in different concentrations on human cultured peripheral lymphocytes, in healthy individuals, using comet assay. The extent of DNA damage reflects a balance between oxidative stress (the presence of hydrogen peroxide H₂O₂ as a reactive oxygen species ROS), and DNA repair ability (the presence of anti-oxidant may be caffeine substances at known concentrations). This is an important method to prevent and avoid many cancerous diseases in an era of various pollutants.

Methods: Ten milliliters of venous blood samples were collected from 40 healthy young individuals, and lymphocyte cultures were set up after lymphocyte isolation with ficoll centrifugation. The mixture of lymphocytes culture media was incubated in the sterile incubator for 5 min after adding serial concentrations of caffeine (100, 500, 5000, 10000) µg/ml, as (group 1, 2, 3, 4 respectively) to 5% H₂O₂. The levels of oxidative DNA damage were expressed as comet tail length.

Results: At concentration 100 µg/ml, there was a significant elevation in the mean comet tail length level in cultured lymphocytes treated with hydrogen peroxide (106.96 µm) compared with the treated with All (mixture of caffeine, and H₂O₂), 6.670 µm.

Conclusion: We've concluded that a caffeine concentration of 100 µg/ml possesses the strongest anti-oxidant properties and causes much less DNA damage in lymphocytic culture when exposed to hydrogen peroxide.

Keywords: Oxidative DNA damage, Comet assay, Lymphocyte, Hydrogen peroxide

© 2016 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

INTRODUCTION

Cultured human lymphocytes are the standard model for individual eukaryotic cells that can be vitally evaluated cytologically by phase contrast and differential contrast microscopy. They can be assessed both cytochemically and biochemically for various perturbation affecting nuclear DNA including various form of DNA damage and repair [1].

Caffeine is the one of the top commonly consumed dietary ingredients in the world. It is naturally available in coffee beans, cacao beans, kola nuts, and in the tea leaves. Studies showed that caffeine may be beneficial in reducing DNA damage in result to UV exposure [1, 2]. Potential anti-inflammatory properties of caffeine have also been studied and concluded that the moderate coffee drinking was associated with an increase in the concentration of several inflammatory biomarkers, including c-reactive protein, interleukin 6, tumor necrosis factor α, and amyloid A compared to non-coffee drinkers [3, 4].

Evaluating the levels of oxidative DNA damage and the role of caffeine is important in studies which assess the degree of genotoxicity, toxicological studies, as well as the occupational exposures [5]. Several methods have been established to assess the levels of DNA damage.

This is mainly based on the type of the damaging agent, the DNA kinetics repairs, measuring end point, and the ways for measuring the endpoint (whether qualitatively or quantitatively). Comet assay is a novel approach for the assessment of DNA strand breakage in a single cell. It is one such state-of-the-art technique for quantifying DNA damage and repair *in vivo* and *in vitro* in any eukaryotic cell and some prokaryotic cells [6]. This is a rapid technique, not-invasive, highly sensitive, visual and inexpensive as compared to the conventional techniques and is a powerful tool to study factors modifying mutagenicity and carcinogenicity [7].

MATERIALS AND METHODS

Human subjects, cell culture, and treatments

Forty healthy individuals (26 female and 14 male) with no recent medical illness have been selected for the study. These participants were age-compatible, non-smokers, non-drinkers. The study also excluded participants with known medical history, dietary supplements or whom taking prescribed medication. This study was approved by the human ethics committee at Al-Nahrain University, Baghdad, Iraq (ethics number MA35).

Ten milliliters of venous blood samples were collected, and lymphocyte cultures were set up after lymphocyte isolation with ficoll centrifugation. The culture medium used was composed of RPMI1640 (CAPRICORN Scientific, Germen), containing 10% fetal calf serum (Sigma), penicillin G solution (final concentration 0.1 mg/ml), L-glutamine (BDH), and streptomycin solution (final concentration 0.1 mg/ml). The lymphocyte suspension was utilized for cell culture according to the procedure described by [8]. The mixture of lymphocytes culture media was incubated in the sterile incubator (Gallen kamp size one, model 1H-150, England), for 5 min after adding different concentrations of caffeine (100, 500, 5000, and 10000) µg/ml, as (group 1, 2, 3, 4 respectively) to 5% H₂O₂; for use in the comet assay.

Preparation of caffeine solutions (Santa Cruz Biotechnology, Inc., sc-202485)

To prepare a stock solution of caffeine, that has a concentration of (10000 µg/ml), weigh a 0.1 gm of the powder and add it into a sterilized test tube containing (10 ml of DMSO solution to form a stock solution of caffeine with a concentration of (10000 µg/ml). Then do a serial dilution to prepare the other concentrations (5000 µg/ml, 500 µg/ml, and 100 µg/ml) solutions by adding 1:1 ratio RPMI media for preparation of the four groups (1, 2, 3, 4 respectively).

Alkaline comet assay (alkaline single-cell gel electrophoresis) [6]

Alkaline single-cell gel electrophoresis (SCGE) was performed in order to detect the levels of genotoxicity in treated and untreated lymphocyte culture in the presence of different concentrations (100, 500, 5000, 10000) $\mu\text{g/ml}$ of caffeine [6]. In brief, lymphocytes were re-suspended in 0.5 ml of phosphate buffered saline (PBS), and 5 μL of cell cultured suspension was mixed with 35 μL of 1% (w/v) low-melting-point agarose (LMPA; Sigma-Aldrich) and added to slides coated with 0.5% (w/v) normal-melting-point agarose (NMPA; Sigma-Aldrich). Cover slips were added and slides were incubated on ice packs until solidification of the agarose. Coverslips were then removed and 40 μL of 1% (w/v) LMPA was added to the slides. Slides were incubated in a lysis solution of (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris; pH 10) at 4 °C in the dark for 2h. Slides were incubated in electrophoresis buffer (300 mM NaOH, 1 mM EDTA disodium salt; pH>13) in the dark for 20 min and electrophoresis was performed at 24 V (300 mA) for 30 min. After neutralization (0.4 M Tris; pH 7.5), slides were stained with 10 mg/ml of ethidium bromide and observed under a fluorescence microscope (Olympus-Japan). A computerized image analysis system (Comet Assay IV, Perceptive Instruments, UK) was employed. Tail length (μm) was used as the measure of DNA damage. A minimum of 4 SCGE slides was prepared for each treatment, and in total, 50 nuclei were analyzed per treatment [9]. The Comet assay measurements, (tail length) in cultured peripheral lymphocytes treated with the different four caffeine concentrations in this study, 5 % of hydrogen peroxide solution was added to the cells for 5 min on ice, controls were exposed to the (solvent and culture media), and All (mixture of caffeine, and hydrogen peroxide). Cultures slides were prepared in parallel; the process was repeated for 40 samples, and for all the groups.

Cell viability assay

The cell viability was evaluated by using methyl thiazolyl tetrazolium bromide (MTT, Sigma, USA) assay. In brief, 100 μL of the MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide solution, 5 mg/ml in PBS were added to each well, which contained 100 μL of the treated cultured lymphocytes. The plates were incubated for 3 h at 37 °C. Then, for solubilization of the MTT crystals, 100 μL of isopropanol (Merck, Germany) was added to the wells. Finally, the optical density of each well was determined with a blank reagent with a multiwell scanning spectrophotometer (ELISA reader, Organon Teknika, Netherlands) at a wavelength of 570 nm. The Percentage of cell viability related to control (untreated) was calculated by $[A]_{\text{test}}/[A]_{\text{control}} \times 100$. Where [A] test is the absorbance of the test sample and [A] control is the absorbance of control sample [10].

Statistical analysis

Data were analyzed using the statistical package of SPSS version 18. Mean and standard error or means were measured for the continuous variables. Analysis of variance (ANOVA) and least significant difference (LSD) between means at level of significance (0.05). All hypothesis testing two-tailed considering a significant P value at or below 0.05. Regression and correlation (r) were calculated to check the relationship between variables and t-test has been used to test the significant differences.

RESULTS

The comet tail length values

The Comet tail length values for cultured lymphocytes treated with the 100 $\mu\text{g/ml}$ concentration of caffeine, and 5% H_2O_2 in the (group 1)

Table 1 shows the results for group 1. There was a significant elevation in the mean comet tail length level in cultured lymphocytes treated with hydrogen peroxide (106.96 μm) compared with the treated with All (mixture of caffeine, and H_2O_2), 6.670 μm , there were no significant correlations between the mean comet tail length level in cultured lymphocytes treated with All (caffeine, H_2O_2), and control, (LSD = 1.047, $P \leq 0.05$).

Table 1: Comet tail length (μm) values of the cultured lymphocytes treated with caffeine, H_2O_2 , all (caffeine, H_2O_2), and controls in group (1)

Treatments	Concentration 100 $\mu\text{g/ml}$ (40 Samples)	
	mean \pm SEM	Sig.
H_2O_2	106.960 \pm 1.146	*
Caffeine	3.980 \pm 0.446	*
ALL(caffeine, H_2O_2)	6.670 \pm 0.426	*
Control	7.600 \pm 0.197	

* Significant differences at P equal or less than 0.05., N=40 samples, Data are given in mean \pm SEM

A picture of single-cell gel electrophoresis (SCGE) to the cultured lymphocytes stained with ethidium bromide and present in different cases of four subgroups of group 1 were shown in fig. (1).

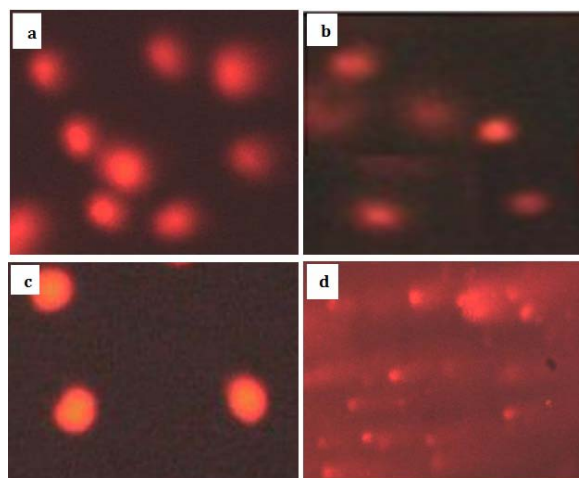


Fig. 1: Photographs of single-cell gel electrophoresis (SCGE) to the cultured lymphocytes present in the four subgroups (a, b, c, d) of group 1 and stained with ethidium bromide, a-Cultured lymphocytes in group 1, b-Cultured lymphocytes in group 1 (caffeine), c-Cultured lymphocytes in group 1 (control), d-Cultured lymphocytes in group 1 (H_2O_2), $\times 400$ (Fluorescent Microscope, Olympus-Japan), N=40 samples, Data given in mean \pm SEM

2-The Comet tail length values for cultured lymphocytes treated with the 500 $\mu\text{g/ml}$ concentration of caffeine and 5% H_2O_2 , in (group 2)

Table 2 shows the results for group 2. There was a highly significant elevation in the mean tail length level in the cultured lymphocytes treated with hydrogen peroxide (116.080 μm) compared with this treated with Caffeine, All (caffeine, H_2O_2) and also with control (LSD = 2.014, $P \leq 0.05$). There was no significant correlation between the mean tail length levels in the cultured lymphocytes treated with caffeine and control, (LSD = 2.014, $P \leq 0.05$).

The Comet tail length values for cultured lymphocytes treated with the 5000 $\mu\text{g/ml}$ concentration of caffeine, and 5% H_2O_2 in group (3)

Table 3 shows the results for group 3. There was a significant elevation in the mean tail length level in the cultured lymphocytes treated with hydrogen peroxide (126.260 μm) compared with this treated with caffeine, and with control (LSD = 2.014, $P \leq 0.05$), also, there was a significant elevation in the mean tail length level in the cultured lymphocytes treated with hydrogen peroxide with these treated with All (Caffeine, and H_2O_2). There was no significant correlation between the mean tail length level in the cultured lymphocytes treated with caffeine and the control, ($P \leq 0.05$).

Table 2: Comet tail length (µm) values of the cultured lymphocytes treated with caffeine, H₂O₂, all (caffeine, H₂O₂), and controls in group (2)

Treatments	Concentration 500 µg/ml (40 Samples)	
	mean±SEM	Sig.
H ₂ O ₂	116.080±0.588	*
Caffeine	6.100±0.412	*
ALL(caffeine, H ₂ O ₂)	20.415±1.753	*
Control	7.175±0.057	

* Significant differences at P equal or less than 0.05., N=40 samples, Data are given in mean±SEM

Fig. 2 shows pictures of single-cell gel electrophoresis (SCGE) stained with ethidium bromide for all the subgroups of group 2.

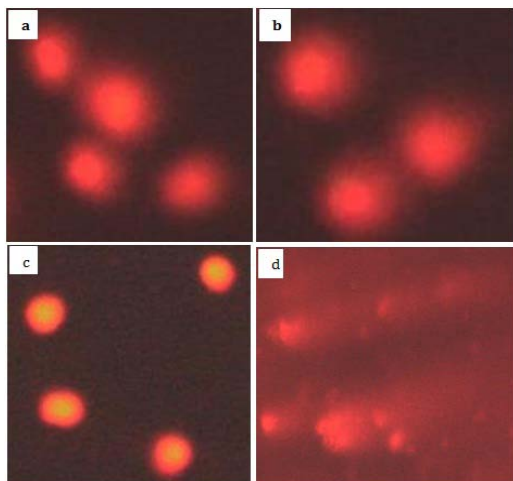


Fig. 2: Photographs of single-cell gel electrophoresis (SCGE) to the cultured lymphocytes present in the four subgroups (a, b, c, d) of group 2 and stained with ethidium bromide, a-Cultured lymphocytes in group 2, b-Cultured lymphocytes in group 2 (caffeine), c-Cultured lymphocytes in group 2 (control), d-Cultured lymphocytes in group 2 (H₂O₂), x400 (Fluorescent Microscope, Olympus-Japan)., N=40 samples, Data given in mean±SEM

Table 3: Comet tail length (µm) values of the cultured lymphocytes treated with caffeine, H₂O₂, all (caffeine, H₂O₂), and controls in group (3)

Treatments	Concentration 5000 µg/ml (40 Samples)	
	mean±SEM	Sig.
H ₂ O ₂	126.260±2.874	*
Caffeine	7.230±0.201	*
ALL(caffeine, H ₂ O ₂)	60.640±1.824	*
Control	8.250±0.151	

* Significant differences at P equal or less than 0.05., N=40 samples, Data are given in mean±SEM

Photographs of single-cell gel electrophoresis (SCGE) of cultured lymphocytes stained with ethidium bromide were presented in fig. 3B and in different cases of a, b, c, d; in (group3)

The Comet tail length values for cultured lymphocytes treated with the 10000 µg/ml concentration of caffeine, and 5% H₂O₂ in group (4)

Table 4 shows the results for all the subgroups of group 4. There was a significant elevation in the mean tail length value in the cultured lymphocytes treated with hydrogen peroxide, (120.750 µm) compared with the treated with caffeine, and with control (LSD =4.295, P ≤ 0.05). Also, there was a highly significant elevation in the mean tail length level in the cultured lymphocytes treated with

hydrogen peroxide, (120.750 µm), and with the treated with All (caffeine, H₂O₂), (73.320 µm). There was a significant correlation between the mean tail length level in cultured lymphocytes treated with caffeine and with the mean tail length level in the control cultured lymphocytes, (12.600 and 7.840) µm, P ≤ 0.05.

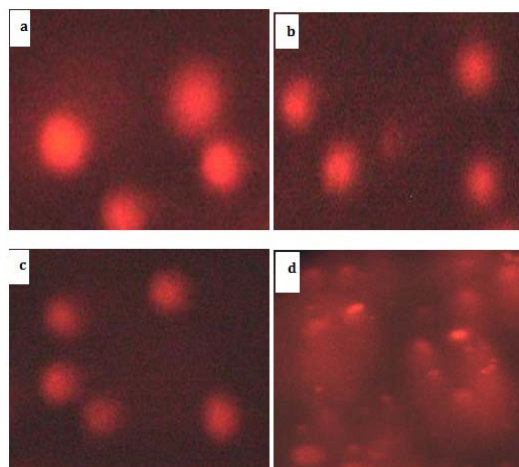


Fig. 3: Photographs of single-cell gel electrophoresis (SCGE) to the cultured lymphocytes present in the four subgroups (a, b, c, d) of group 3 and stained with ethidium bromide, a-Cultured lymphocytes in group 3, b-Cultured lymphocytes in group 3 (caffeine), c-Cultured lymphocytes in group 3 (control), d-Cultured lymphocytes in group 3 (H₂O₂), x400 (Fluorescent Microscope, Olympus-Japan)., N=40 samples, Data given in mean±SEM

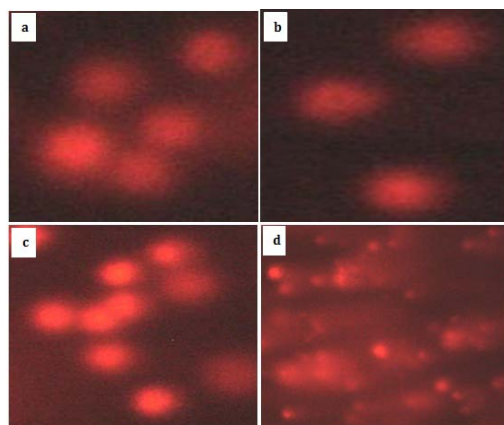


Fig. 4: Photographs of single-cell gel electrophoresis (SCGE) to the cultured lymphocytes present in the four subgroups (a, b, c, d) of group 4 and stained with ethidium bromide, a-Cultured lymphocytes in group 4, b-Cultured lymphocytes in group 4 (caffeine), c-Cultured lymphocytes in group 4 (control), d-Cultured lymphocytes in group 4 (H₂O₂), x400 (Fluorescent Microscope, Olympus-Japan)., N=40 samples, Data given in mean±SEM

Table 4: Comet tail length (μm) values of the cultured lymphocytes treated with caffeine, H_2O_2 , all (caffeine, H_2O_2), and controls in group (4)

Treatments	Concentration 10000 $\mu\text{g}/\text{ml}$ (40 Samples)	
	mean \pm SEM	Sig.
H_2O_2	120.750 \pm 1.495	*
Caffeine	12.600 \pm 1.131	*
ALL(caffeine, H_2O_2)	73.320 \pm 3.646	*
Control	7.840 \pm 0.281	*

* Significant differences at P equal or less than 0.05., N=40 samples, Data are given in mean \pm SEM

Pictures of single-cell gel electrophoresis (SCGE) stained with ethidium bromide, were showed in fig. (4); (a, b, c, and d), in group 4.

DISCUSSION

In living system, oxidative stress produces reactive oxygen species (ROS) like superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{HO}\cdot$). H_2O_2 spontaneously converted to the highly reactive hydroxyl radicals ($\text{HO}\cdot$), these hydroxyl radicals oxidize proteins, lipids and nucleic acids leading to mutations at the cellular levels [11].

This study investigated the degree of oxidative DNA damage in normal lymphocytic culture, in the presence of caffeine, vs. hydrogen peroxide (H_2O_2) alone or in mixed, *in vitro* experiments was carried out. Our study showed that exposure of the cells to different concentrations of caffeine (100, 500, 5000, 10000) $\mu\text{g}/\text{ml}$, as the four groups of the study are not toxic to cultured human lymphocytes (based on both comet assay and on MTT assay), but they acted as an antioxidant against hydrogen peroxide (H_2O_2), as showed in our results. This is in agreement with those of [12, 13], who suggested that during *in vitro* experiments, there were protective effects of antioxidants, and there were inhibitory effects of caffeine on the radicals-mediated oxidation and mutagenicity. The comet assay which is used in this study were adequately detecting significant differences in single strand breaks between normal human cultured lymphocytes treated with beta-carotene, caffeine and treated with H_2O_2 .

Comet tail length in group 1 (caffeine 100 $\mu\text{g}/\text{ml}$)

There was a significant positive correlation between the cultured lymphocytes treated with caffeine and the control of cultured lymphocytes via assessing the comet tail length. This means that the behavior of the caffeine as anti-oxidant reagent toward the cultured lymphocytes is best at a concentration of 100 $\mu\text{g}/\text{ml}$.

These findings were in agreement with Bichler et al. [14], who suggested that coffee consumption may prevent oxidative DNA-damage to a higher extent as diet enriched with fruits and vegetables. In the mixture of caffeine and H_2O_2 (All) treatments to cultured lymphocytes, the comet tail length was negatively correlated with H_2O_2 treatment. This means that caffeine can act as an antioxidant best at a concentration of 100 $\mu\text{g}/\text{ml}$. This was also supported by [15], who found that coffee consumption caused protective effects, and it is notable that it was more effective than the consumption of a diet containing increased levels of fruits and vegetables.

Comet tail length in group 2 (caffeine 500 $\mu\text{g}/\text{ml}$)

There was no significant correlation between cultured lymphocytes treated with caffeine and the control of cultured lymphocytes in comet tail length, meaning that caffeine possesses less anti-oxidant effects at this level. In the mixture caffeine and H_2O_2 (All treatment to cultured lymphocytes), the comet tail length was less significantly correlated with H_2O_2 treatment of the cultured lymphocytes (compared with the mixture in group 1). Reactive oxygen species, when present in the body, can damage the normal cellular functions and can cause atherosclerosis as well as malignant growth in some tissues as well as they can contribute to the ageing process [16]. Our study showed a significant DNA damage (by a significant increase in the tail length of DNA of cultured lymphocytes treated with H_2O_2), when compared the control, this finding is in agreement with [17],

who observed that the lymphocytes when treated with H_2O_2 showed a significant DNA damage.

Comet tail length in group 3 (caffeine 5000 $\mu\text{g}/\text{ml}$)

There was a non-significant correlation between the cultured lymphocytes treated with caffeine and the lymphocytic control culture. A finding similar to a 500 $\mu\text{g}/\text{ml}$ concentration. In the mixture of caffeine and H_2O_2 (All treatments to cultured lymphocytes), the comet tail length was less correlated with H_2O_2 treatment of the cultured lymphocytes (compared with the All in group2), this agreement with the earlier study done by [18], which claimed a protective role of glutathione included caffeine against oxidative DNA damage. Glutathione has several important functions, including protection against oxidative stress, increased the DNA-repairs, regulation of gene expression, induction of apoptosis, and activation and proliferation of lymphocytes [19].

Comet tail length in group 4 (caffeine 10000 $\mu\text{g}/\text{ml}$)

There was a significant negative correlation between the cultured lymphocytes treated with caffeine and the control of cultured lymphocytes in comet tail length. This indicates a pro-oxidant role of caffeine at higher doses. This finding is in agreement with Gülçin I [20], who showed that caffeine possesses pro-oxidant properties under high doses.

CONCLUSION

From our study, we've concluded that a caffeine concentration of 100 $\mu\text{g}/\text{ml}$ possesses the strongest anti-oxidant properties and causes much less DNA damage in lymphocytic culture when exposed to hydrogen peroxide.

CONFLICT OF INTERESTS

Declared none

REFERENCES

- Abel E, Hendris S, McNeeley S, Johnson K, Rosenberg C, Mossavar-Rahmani Y, Vitols M, Kruger M. Daily coffee consumption and prevalence of nonmelanoma skin cancer in white Caucasian women. *Eur J Cancer Prev* 2007;16:446-52.
- Kerzendorfer C, O'Driscoll. UVB and caffeine: inhibiting the DNA damage response to protect against the adverse effects of UVB. *J Invest Dermatol* 2009;129:1611-3.
- Zampelas A, Panagiotakos DB, Pitsavos C, Chrysohoou C, Stefanadis C. Associations between coffee consumption and inflammatory markers in healthy persons: the ATTICA study. *Am J Clin Nutr* 2004;80:862-7.
- Snel J, Lorist MM. Effects of caffeine on sleep and cognition. *Prog Brain Res* 2011;190:105-17.
- Stanner SA, Hughes J, Kelly CN, Buttriss J. A review of the epidemiological evidence for the antioxidant hypothesis public. *Health Nutr* 2004;7:407-22.
- Nandhakumar S, Parasuraman S, Shanmugam MM, Ramachandra Rao K, Chand P, Bhat BV. Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay). *J Pharmacol Pharmacother* 2011;2:107-11.
- Maier BJ, Hofer E, Carin C, Lhoste E, Uhl M, Glatt HR, et al. Coffee diterpenes prevent the genotoxic effects of 2-amino-1-methyl-6-N-nitroso dimethylamine in human derived liver cell line (Hep G2). *Food Chem Toxicol* 2005;43:433-41.
- Potter A, Kim C, Gallahon KA, Rabinovitch PS. Apoptotic human lymphocytes have diminished CD4 and CD8 receptor expression. *Cell Immunol* 1999;193:36-47.
- O'Brien NM, Woods JA, Aherne SA, O'Callaghan YC. Cytotoxicity, genotoxicity and oxidative reactions in cell-culture models: modulatory effects of phytochemicals. *Biochem Soc Trans* 2000;28:22-6.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
- Halliwell B, Gutteridge JM. Free radicals in biology and medicine. 4th Edition. Oxford University Press, USA; 2007.
- Stadler RH, Markoric J, Turesky RJ. *In vitro* anti- and pro-oxidative effects of natural polyphenols. *Biol Trace Elem Res* 1995;47:299-305.

13. Stadler RH, Turesky RJ, Müller O, Markovic J, Leong-Morgenthaler PM. The inhibitory effects of coffee on radical-mediated oxidation and mutagenicity. *Mutat Res* 1994; 308:177-90.
14. Bichler J, Cavin C, Simic T, Chakraborty A, Ferik F, Hoelzl C, *et al.* Coffee consumption protects human lymphocytes against oxidative and 3-amino-1-methyl-5H-pyrido[4,3-b]indole acetate (Trp-P-2) induced DNA-damage: results of an experimental study with human volunteers. *Chem Toxicol* 2007;45:1428-36.
15. Hoelzl C, Knasmüller S, Misík M, Collins A, Dusinská M, Nersesyan A. Use of single cell gel electrophoresis assays for the detection of DNA-protective effects of dietary factors in humans: Recent results and trends. *Mutat Res* 2009;681:68-79.
16. Merzenich UG, Zeitler H, Vetter H, Kraft K. Synergy research: Vitamins and secondary plant components in the maintenance of the redox homeostasis and cell signaling. *Phytotherapy* 2008;16:2-16.
17. Swaczynova J, Malikova J, Hoffmannova L, Kohout L, Strnad M. Anticancer properties of brassinosteroids. *Phytochemistry* 2008;69:418-26.
18. Esposito F, Morisco F, Verde V, Ritieni A, Alezio A, Caporaso N, *et al.* Moderate coffee consumption increases plasma glutathione but not homocysteine in healthy subjects. *Aliment Pharmacol Ther* 2003;17:595-601.
19. Alidoost F, Gharagozloo M, Bagherpour B, Jafarian A, Sajjadi SE, Hourfar H, *et al.* Effects of silymarin on the proliferation and glutathione levels of peripheral blood mononuclear cells from beta-thalassemia major patients. *Int Immunopharmacol* 2006;6:1305-10.
20. Gülçin I. *In vitro* prooxidant effect of caffeine. *J Enzyme Inhib Med Chem* 2008;23:149-52.