Journal of Nutrition & Food Sciences
ISSN: 2155-9600

Zaidi and Rawat, J Nutr Food Sci 2012, 2:6
DOI: 10.4172/2155-9600.1000149

Research Article Open Access

Evaluation of Cytotoxicity of Food in Human Hepatoma HepG2 Cells: Comet Assay Coupled to the MTT Assay

Rana Zaidi* and Pinki Rani Rawat

Department of Biochemistry, Faculty of Science, Jamia Hamdard, New Delhi-110062, India

Abstract

The cooking of meat has been found to generate compounds that possess extreme mutagenicity in short term tests. Heterocyclic aromatic amines are potent bacterial and eukaryotic cell mutagens. In this study we employed two in vitro techniques, the MTT cell viability assay and the single cell gel electrophoretic assay to evaluate food genocytotoxicity in human hepatoma hepG2 cells in home cooked and commercially available food sources. Both representatitve assays confirm that PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine) and MeIQx (2-amino-3,8 dimethylimidazo [4,5-f]quinoxaline) are potent DNA damaging agents in the selected cell line (HepG2). This study correlates the effects of exposure of food carcinogens to humans; we further propose such studies would lead to a better understanding of the risks involved for prevention of liver carcinomas.

Keywords: DNA damage; HepG2 cells; Food genocytotoxicity; Comet assay

Abbreviations: PhIP: 2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine; MeIQx: 2-amino-3,8 dimethylimidazo[4,5-f]quinoxaline

Introduction

Hitherto approximately twenty five different mutagenic compounds [1,2] have been observed to be produced during cooking of protein rich foods, especially meats which could be carcinogenic/mutagenic to humans. In recent years, considerable effort has been made to characterize, identify and quantitate these compounds in human foods and to elucidate their potential health risks [3,4] .Heterocyclic aromatic amines have been tested extensively in microbial in vitro assays such as Ames Salmonella typhimurium test [5] and are potent mutagens in these test procedures [6] however, literature survey has indicated that almost none of the studies have simultaneously employed two in vitro genocytotoxicity assays, the MTT assay and the comet assay (single cell gel electrophoresis assay) to authenticate the genotoxicity of food.

Further, it is well documented that HepG2 cell lines retain the activity of certain phase I enzymes involved in metabolism of genotoxic carcinogens such as cytochrome P450 CYP1A1, CYP1A2, CYP2B, CYP2E1 as well as phase II enzymes including glutathione-S-transferases, sulfotransferases, n-acteyltransferases and glucoranosyltransferases, which reflect the metabolism of heterocyclic aromatic amines [7] in mammals better than other in vitro models which require addition of exogenous activation mixtures [8]. The potential hepatotoxicity of heterocyclic aromatic amines is supported by experimental evidence [9].

MTT assay is widely used to measure cell proliferation and for screening of anticancer drugs [10]. It is based on the reduction of tetrazolium salt, MTT (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) by actively growing cells to produce a purple formazan product.

A number of studies have been conducted in the past to demonstrate mutagenicity of standard known heterocyclic aromatic amines through various techniques [11-14]. The goal of this study was to investigate food genotoxicity for the first time employing two in vitro cytotoxicity assays via: the MTT assay and the comet assay. Previous studies in our laboratory have established the identification of two potentially carcinogenic food mutagens PhIP (2-amino -1-methyl -6-phenylimidazo [4,5-b]pyridine and MeIQx (2-amino-3,8 dimethylimidazo[4,5-f] quinoxaline in at least eight selected meat samples, four of which were home cooked and the other four, commercially available meat foods [15] for which we employed two techniques i.e., ultraperformance liquid chromatography and mass spectrometry. Amounts detected were in ng/gm. Reports have established PhIP and MeIQx to be the most potential DNA damaging agents [16,17]. Therefore, we were interested to explore whether the selected samples were able to give rise to DNA damage. Comet assay is a simple technique for sensitive and reliable detection of DNA damage in individual cells [18,19] . Since this assay is less time consuming than other genocytotoxicity assays, we anticipate that it might be a suitable approach to investigate DNA damage of heterocyclic amines in the human derived, metabolically competent cell line HepG2 [20]. Such studies would help us understand cancer and its prevention for the future.

Materials and Methods

Chemicals

Amberlite XAD-2 resin was purchased from Supelco (Bellefonte, Pennsylvania, USA). Hydrochloric acid, sodium hydroxide, sodium chloride, agarose, triton X-100, acetone, ethidium bromide were procured from S. D. Fine Chemicals (Mumbai, India). Methanols, benzene, EDTA, dimethyl sulphoxide were purchased from Sisco Research Laboratories (Mumbai, India). Ammonia solution was procured from Merck Chemicals (Mumbai, India). Tris buffer was purchased from Spectrochem (Mumbai, India). Thiazolyl blue tetrazolium bromide (MTT) was procured from Sigma (Saint Louis, USA). All reagents used were of analytical grade.

***Corresponding author:** Rana Zaidi, Department of Biochemistry, Faculty of Science, Jamia Hamdard, New Delhi-110062, India, Tel: +091-11-26059688 ext. 5518; E-mail: ranaamu@yahoo.com

Received May 29, 2012; **Accepted** June 22, 2012; **Published** June 27, 2012

Citation: Zaidi R, Rawat PR (2012) Evaluation of Cytotoxicity of Food in Human Hepatoma HepG2 Cells: Comet Assay Coupled to the MTT Assay. J Nutr Food Sci 2:149. doi:10.4172/2155-9600.1000149

(a) Commercial samples

Sample	Amounts used (µl)	Tail DNA (%)	Tail length (µm)	Olive tail moment
Control (DMSO)	2	2.615 ± 1.25	7.62 ± 1.877	0.74 ± 0.5665
Chicken Kabab	0.5	12.15 ± 3.22*	18.57 ± 3.541**	2.24 ± 1.509^{ns}
	1	35.66 ± 3.942***	66.51 ± 4.215***	11.58 ± 2.048***
	1.5	56.155 ± 3.724***	73.97 ± 4.372***	19.85 ± 3.933***
	2	53.905 ± 3.422***	65.635 ± 3.93***	16.02 ± 1.825***
Chicken Nugget	0.5	66.765 ± 4.105***	72.145 ± 4.109***	22.995 ± 2.101***
	1	64.61 ± 3.194***	64.76 ± 3.644***	23.375 ± 1.565***
	1.5	52.315 ± 3.231***	60 ± 4.459***	18.625 ± 1.913***
	2	53.855 ± 1.892***	54.285 ± 1.048***	17.845 ± 0.7454***
Sardine in olive oil	0.5	57.755 ± 2.104***	67.22 ± 2.125***	22.125 ± 1.337***
	1	51.44 ± 2.306***	58.095 ± 2.242***	17.64 ± 0.9993***
	1.5	57.63 ± 2.013***	65.32 ± 2.336***	20.525 ± 1.122***
	2	54.4 ± 1.572***	62.14 ±1.259***	21.585 ± 0.8306***
Sardine in tomato sauce	0.5	44.19 ± 3.638***	46.27 ± 2.949***	10.64 ± 1.345***
	1	54.03 ± 3.143***	55.4 ± 2.942***	17.32 ± 1.552***
	1.5	51.27 ± 2.525***	55.395 ± 2.171***	17.925 ± 1.272***
	2	59.005 ± 2.927***	67.38 ± 3.128***	21.675 ± 1.444***

(b) Home cooked samples

Sample	Amounts used (µl)	Tail DNA (%)	Tail length (µm)	Olive tail moment
Control (DMSO)	2	2.615 ± 1.25	7.62 ± 1.877	0.74 ± 0.5665
Fried Chicken	0.5	54.24 ± 2.474***	58.02 ± 2.016***	18.61 ± 1.333***
	1	49.63 ± 3.665***	64.68 ± 2.883***	15.905 ± 1.831***
	1.5	60.07 ± 3.227***	79.445 ± 2.383***	23.035 ± 1.712***
	2	71.92 ± 3.036***	84.125 ± 2.166***	31.57 ± 3.87***
Fried Fish	0.5	14.57 ± 2.615**	18.25 ± 3.634***	2.28 ± 1.162^{ns}
	1	44.32 ± 2.786***	64.84 ± 3.837***	11.165 ± 1.529***
	1.5	63.625 ± 4.014***	70.24 ± 3.574***	23.05 ± 2.001***
	2	71.705 ± 2.147***	87.06 ± 1.439***	30.52 ± 1.454***
Fried Mutton	0.5	13.095 ± 2.753*	9.21 ± 2.971ns	1.735 ± 1.104ns
	1	18.105 ± 3.548***	17.38 ± 3.874**	3.105 ± 1.668ns
	1.5	23.135 ± 3.752***	16.115 ± 4.384***	2.94 ± 1.905*
	2	57.37 ± 3.867***	69.84 ± 4.133***	22.03 ± 2.121***
Fried Beef	0.5	26.275 ± 2.506***	22.855 ± 2.704*	4.705 ± 0.7345ns
	1	63.055 ± 3.916***	78.57 ± 3.439***	23.405 ± 2.045***
	1.5	68.695 ± 3.506***	71.59 ± 2.668***	25.065 ± 1.769***
	2	72.695 ± 3.726***	80.71 ± 2.579***	31.02 ± 2.036***

Table 1: Effect of food carcinogens on comet assay parameters for HepG2 cells for a) commercial and b) home cooked samples analyzed in HepG2 cells by comet assay. Data is represented as median ± SEM.

For cell culture experiments, trypsin-EDTA solution, antibiotic-antimycotic solution, Dulbecco's phosphate buffer saline, fetal bovine serum, low melting agarose were purchased from Himedia (Mumbai, India).

Sample preparation and extraction of heterocyclic aromatic amines

All the raw boneless meat samples (beef, mutton, chicken and fish) and commercial foods (chicken kabab, chicken nuggets, sardine in tomato sauce and sardine in olive oil) were procured from local standard markets in New Delhi. Samples were either deep fried in a large volume of Dhara refined oil (commercially available refined mustard oil) or were ready to be consumed as such.

Mutagens were extracted according to the method of [21] with certain modifications [22]. Extracts were evaporated to dryness in a vacuum rotary evaporator and resuspended in a volume of 200µl dimethyl sulphoxide (DMSO) for further use.

Evaluation of genotoxicity of meat foods

HepG2 cells: culture and maintenance: The cells were grown in DMEM supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic solution in culture flasks in an incubator in CO_2 atmosphere (5%) at 37°C (relative humidity 96%).Cell viability and cell counting was done by the trypan blue dye cell viability assay.

Single cell gel electrophoresis assay/ Comet assay

HepG2 cells were suspended in phosphate buffered saline (PBS)

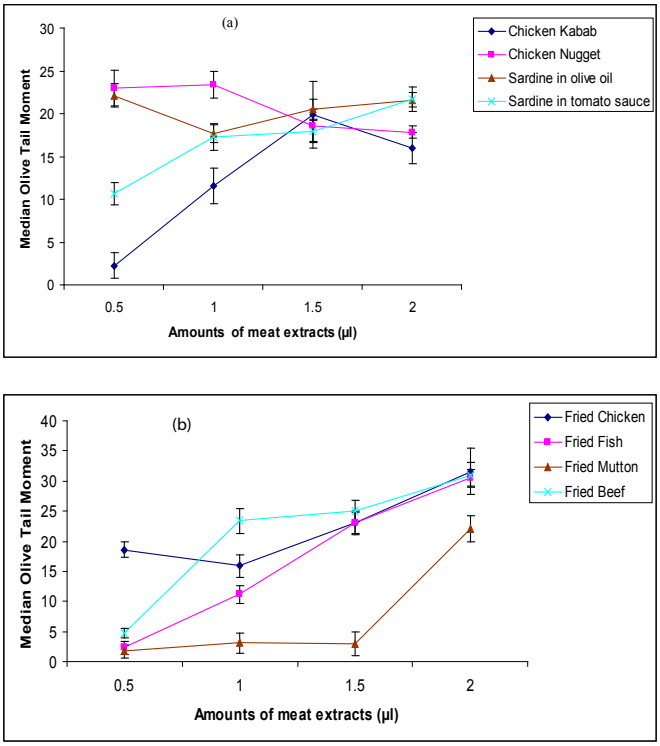


Figure 1: Dose response graphs representing the effect of food carcinogens on the olive tail moment of the comet with increasing doses of the extracts (a) commercial and (b) home cooked samples as analyzed by the comet assay.

(10^5cells/75µl) and incubated with varying amounts of the meat extracts for one hour. In the control incubations, cells were treated with DMSO alone [23]. 200µl of a 1% normal melting point agarose was spread over the frosted microgel electrophoresis slide and kept at room temperature until solidified. Further, the experiment was carried out under dim light conditions. HepG2 cells were mixed with 75µl of 1% low melting point agarose (LMPA). This cell agarose mixture was then poured over the slide, overlayed with a cover glass and kept over ice for 5 min. Cover glass was removed gently and a third layer of 0.5% LMPA was coated and overlayed with a cover glass and kept over ice again for 5 min to solidify. Slides were submerged in cold lysis solution (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM tris, 8 gm NaOH, pH 10; then add 1% triton X-100 and 10% DMSO prior to use) for one hour. Lysis and electrophoresis were performed at 4°C in a cold room. Electrophoresis reservoir was filled with chilled electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 12.3), slides were allowed to submerge for 20 min for unwinding of DNA before executing electrophoresis (24V, 300mA). The slides were removed, drained and coated drop wise with a neutralizing buffer (0.4 M tris, pH 7.5) before further incubation for 5 min. The process was repeated twice. All slides were then stained with 50µl ethidium bromide (20µg/ml) and visualized immediately under an Olympus fluorescence microscope, images taken at 60× magnification. The assay was performed in duplicates for each concentration and a total of 50 images (25 from each replicate slide) were analyzed. Analysis of the data was achieved using Komet 5.5 software from Kinetic Imaging (Andor Bioimaging).

MTT cell viability assay

Cell viability was assessed using the MTT colorimetric assay: MTT salt was initially dissolved in PBS at 5mg/ml and filtered to sterilize and remove insoluble residues. The assay was conducted as per the method of [24] with slight modification. HepG2 cells (50,000cells/well) were seeded onto a 96 well plate and incubated for 18 and 24 hours respectively with different amounts of the sample extracts. Each amount of meat extract was added in triplicates. After incubating the samples for 18 and 24 hours respectively, the plate was centrifuged at 1250 rpm at 4°C for 5 minutes, media removed and cells washed with 1x PBS. Then 50µl of MTT (5mg/ml) was added to each well under dim light, covered with aluminum foil and incubated for another five hours. Further, 100µl of DMSO was added to each well and again incubated for one hour. Finally, absorbance was recorded at 550 nm on an ELISA reader.

Results and Discussion

Table 1 represents the comet assay data for commercial meat samples (a) and home cooked meat samples (b) analyzed by Komet 5.5 software. Results represented here are tail DNA (%), tail length (µm) and olive tail moment of the comets. Experimental controls (DMSO) were simultaneously run for both, commercial and home cooked samples. Results obtained in Table 1a indicate that for 0.5µl chicken kabab, the calculated olive tail moment was not significant, whereas for 1, 1.5 and 2µl amounts of the same sample we obtained significant differences in values when compared to controls. Other commercial meat samples gave similar responses. Results in table1b represent similar results when home cooked samples were analyzed: for the minimum amounts used, the olive tail moments were not significant except for that of fried chicken and fried fish respectively. Previous studies [15] have shown that the total amounts of representative food mutagens (PhIP and MeIQx=325.83ng/gm) was found to be highest in fried fish which support the obtained results, although all home cooked meats indicate

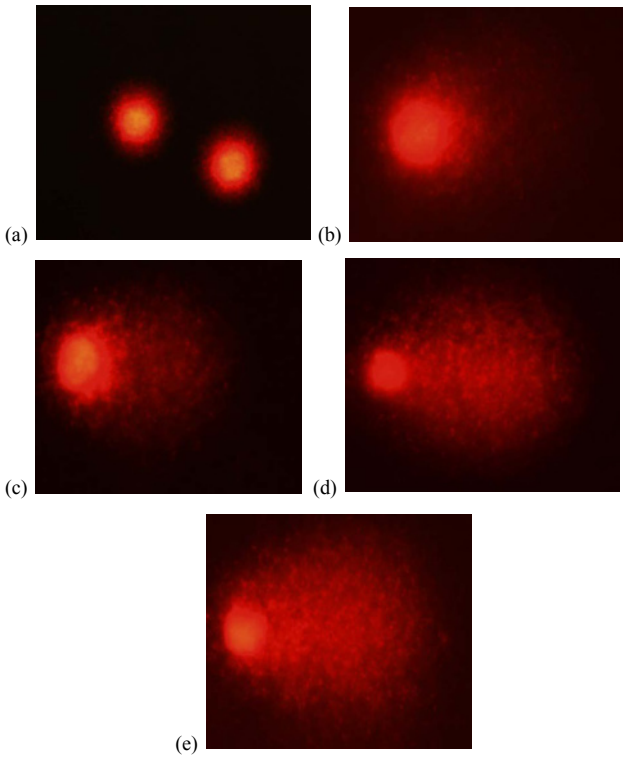
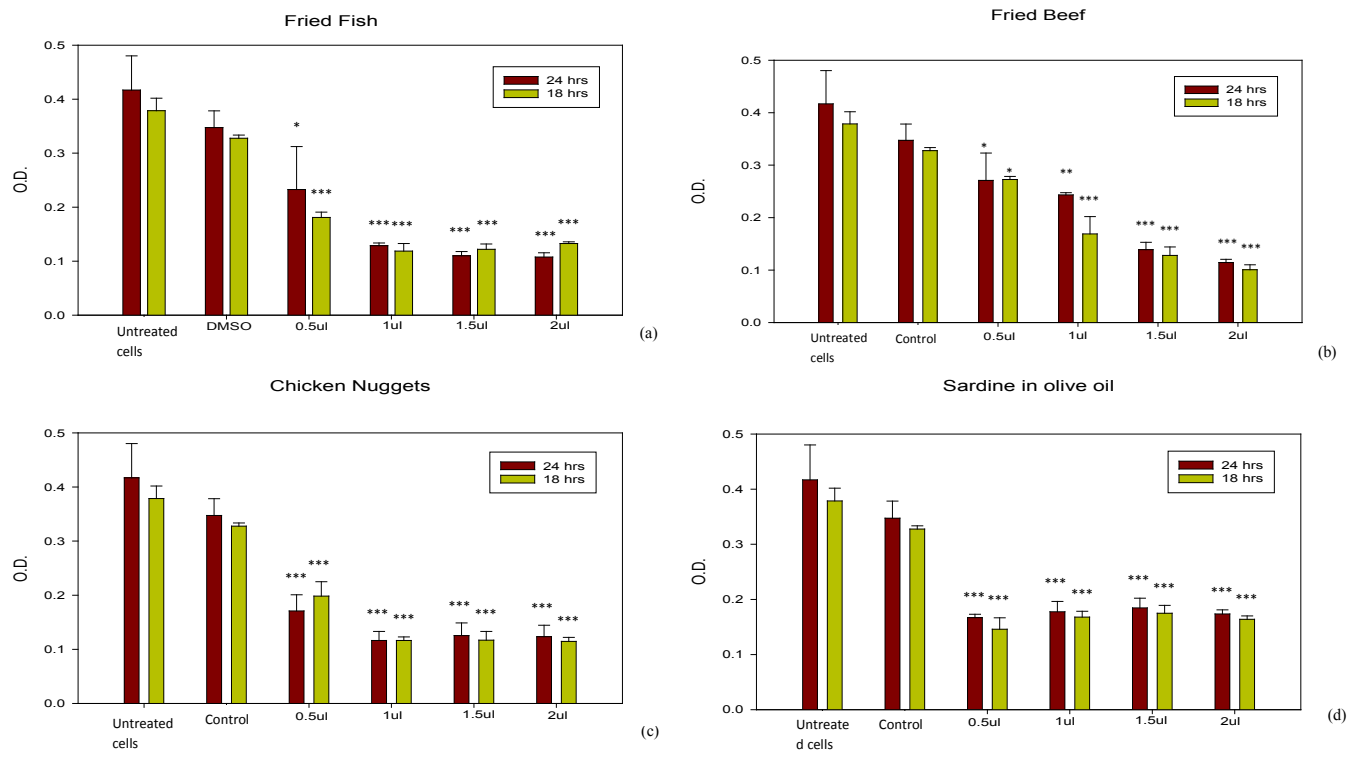


Figure 2: Images of comets with (a) Dimethyl sulfoxide alone as a control and increasing amounts of fried fish extracts (b) 0.5µl (c) 1µl (d) 1.5µl and (e) 2µl respectively.



Data is represented as the mean ± SD (n = 3).
***, significantly different when compared to controls ($p < 0.001$)
**, significantly different when compared to controls ($p < 0.01$)
*, significantly different when compared to controls ($p < 0.05$)

Figure 3: Bar graphs indicating the results of MTT cell viability assay after 18 and 24 hrs treatment of HepG2 cells with various sample extracts (a) fried fish (b) fried beef (c) chicken nuggets and (d) sardine in olive oil.

that the damage increases with an increase in the sample amounts. Figure 1 represents the dose response graph of (a) home cooked and (b) commercially obtained samples, as selected for the present study. The extent of damage observed was higher with home cooked deep fried samples as compared to the commercial samples analyzed. The observed pattern of DNA damage, as a function of median olive tail moment of the comet was found to be maximum in sardine in tomato sauce, followed by chicken kabab. Sardine in olive oil and chicken nuggets almost gave similar dose response results that are represented in Figure 1a. In Figure 1b, when home cooked samples were analyzed, the best response was observed in fried fish, fried chicken and fried beef. Damage was observed at higher amounts for fried mutton, a steep rise in the olive tail moment of the comet was observed at a 2µl amount of the sample used. We hereby conclude that for the home cooked samples analyzed by us the extent of damage was higher as compared to commercial meat samples.

Figure 2 represents the images of comet which were obtained and analyzed by adding fried fish extract to the HepG2 cells. These images clearly indicate and authenticate that when cells were exposed to the extract, the percentage of DNA in the tail of the comet as well as the length of the tail was substantially increased. With increasing doses, more material moved out from the comet head as shown in the representative figure, alternatively no tail was observed in the control when DMSO alone was incubated with the HepG2 cells.

All the eight samples were subsequently evaluated for cytotoxicity by the MTT assay, as previously described in the methodology section. Four different amounts (0.5, 1.0, 1.5 and 2µl) were run in triplicates and incubated for the required time intervals of 18 and 24 hours respectively. Vehicle (DMSO) alone was added in the controls. The data represented in Figure 3 clearly indicates that in almost all of the four selected samples i.e., fried fish, fried beef, chicken nuggets and sardines in olive oil we observe an amount dependent /dose dependent increase in cell death.

Concluding remarks

We employed the comet assay for evaluating genotoxic effects of food in HepG2 cells. The in vitro genotoxicity assay (comet assay) and MTT (cytotoxicity assay) when performed, revealed that almost all samples when tested were found to cause DNA damage and cell death in a dose dependent manner. Comparative analysis of home cooked and commercial sample types employing these assays has revealed that a higher extent of damage was observed with home cooked samples. Using the MTT assay, we demonstrate that, in general, the cytotoxic effects of fried meat extracts correlated with the extent of DNA damage observed in the HepG2 cells.

Statistical analysis

Values are represented as mean ± SD (n=3) and statistical analysis was performed using Graph Pad In Stat software. As the treated groups were compared with control, a one way analysis ANOVA was followed by Dunnett's test and statistical differences at $p < 0.05$ were found to be significant.

Acknowledgements

The authors are thankful to Om Singh for support during the cell culture work. University Grants Commission, New Delhi is duly acknowledged for providing financial assistance to this major research project F. No. 33-221/2007 (SR). Sudhir Chandna is acknowledged for assistance in comet analysis.

References

1. Felton JS, Knize MG (1991) Occurrence, identification, and bacterial mutagenicity of heterocyclic amines in cooked food. Mutat Res 259: 205-217.
2. Sugimura T (1985) Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process. Mutat Res 150: 33-41.
3. Hatch FT, Knize MG, Healy SK, Slezak T, Felton JS (1988) Cooked-Food Mutagen Reference List and Index. Environ Mol Mutagen 12: 1-85.
4. Overvik E, Gustafsson JA (1990) Cooked-food mutagens: current knowledge of formation and biological significance. Mutagenesis 5: 437-446.
5. Gooderham NJ, Murray S, Lynch AM, Yadollahi-Farsani M, Zhao K, et al. (2001) Food-derived heterocyclic amine mutagens: variable metabolism and significance to humans. Drug Metab Dispos 29: 529-534.
6. de Meester C (1989) Bacterial mutagenicity of heterocyclic amines found in heat-processed food. Mutat Res 221: 235-262.
7. Knasmüller S, Parzefall W, Sanyal R, Ecker S, Schwab C, et al. (1998) Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. Mutat Res 402: 185-202.
8. Chang G, Jacobson-Kram D, Williams JR (1988) Use of an established human hepatoma cell line with endogenous bioactivation for gene mutation studies. Cell Biol Toxicol 4: 267-279.
9. Snyderwine EG, Davis CD, Nouso K, Roller PP, Schut HA (1993) 32P-postlabeling analysis of IQ, MeIQx and PhIP adducts formed in vitro in DNA and polynucleotides and found in vivo in hepatic DNA from IQ-, MeIQx- and PhIP-treated monkeys. Carcinogenesis 14: 1389-1395.
10. Maioli E, Torricelli C, Fortino V, Carlucci F, Tommassini V, et al. (2009) Critical appraisal of the MTT assay in the presence of rottlerin and uncouplers. Biol Proced Online 11: 227-240.
11. Knasmüller S, Schwab CE, Land SJ, Wang CY, Sanyal R, et al. (1999) Genotoxic effects of heterocyclic aromatic amines in human derived hepatoma (HepG2) cells. Mutagenesis 14: 533-540.
12. Pfau W, Martin FL, Cole KJ, Venitt S, Phillips DH, et al. (1999) Heterocyclic aromatic amines induce DNA strand breaks and cell transformation. Carcinogenesis 20: 545-551.
13. Uhl M, Helma C, Knasmüller S (2000) Evaluation of the single cell gel electrophoresis assay with human hepatoma (Hep G2) cells. Mutat Res 468: 213-225.
14. Maron DM, Ames BN (1983) Revised methods for the Salmonella mutagenicity test. Mutat Res 113: 173-215.
15. Zaidi R, Rawat P R (2011) Identification of heterocyclic amines in Indian home cooked and commercially available meat foods. J Nutr Food Sci 1: 1-7.
16. http://ntp.niehs.nih.gov/ntp/newhomeroc/roc11/HCAsPub.pdf
17. IARC (1993) Heterocyclic aromatic amines. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines, and Mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans. (Vol.56) IARC, Lyon, France.
18. Klaude M, Eriksson S, Nygren J, Ahnström G (1996) The comet assay: mechanisms and technical considerations. Mutat Res 363: 89-96.
19. Fairbairn DW, Olive PL, O'Neill KL (1995) The comet assay: a comprehensive review. Mutat Res 339: 37-59.
20. Uhl M, Helma C, Knasmüller S (1999) Single-cell gel electrophoresis assays with human-derived hepatoma (HepG2) cells. Mutat Res 441: 215-224.
21. Bjeldanes LF, Grose KR, Davis PH, Stuermer DH, Healy SK, et al. (1982) An XAD-2 resin method for efficient extraction of mutagens from fried ground beef. Mutat Res 105: 43-49.
22. Perez C, Lopez de Cerain A, Bello J (2002) Modulation of mutagenic activity in meat samples after deep-frying in vegetable oils. Mutagenesis 17: 63-66.
23. Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175: 184-191.
24. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.