Appendices

Appendix 1

Questionnaire on Air Quality Monitoring

S.NO	PARTICULARS
1.	Location of the target site
2.	Number of Photocopier machines
3.	Other Volatile Organic compounds emitting sources- printer, carpets, fax machine, incense
4.	Photocopier Speed
5.	Toner Brand: Type: Colour/Black / White Category: Dry / Wet
6.	Number of copies made per day
7.	Position
8.	Room Dimension
9.	Interior floor – ceramic, carpet
10.	Roof
11.	Walls
12.	Doors
13.	Furniture
14.	Air conditioner (Yes / No)
15.	Number of entrances
16.	Business hours

Signature of the Proprietor of the photocopy shop

Appendix 2

Estimation of Fine Particulate Matter ($PM_{2.5}$) in Ambient Air
Gravimetric Method
(BIS 5182 : P4, 2005).

Principle

It is based on gravimetric method that is by weighing the mass of the particles. $PM_{2.5}$ refers to fine particles that are 2.5 µm or smaller in diameter.

Procedue

An electrically powered air sampler draws ambient air at a constant volumetric flow rate (16.67 l/m) maintained by a mass flow / volumetric flow controller coupled to a microprocessor into specially designed inertial particle-size separator where the suspended particulate matter in the $PM_{2.5}$ size ranges is separated for collection on a 47 mm polytetrafluoroethylene (PTFE) filter over a specified sampling period. Each filter is weighed before and after sample collection to determine the net gain due to the particulate matter. The mass concentration in the ambient air is computed as the total mass of collected particles in the $PM_{2.5}$ size ranges divided by the actual volume of air sampled, and is expressed in µg / m^3. The microprocessor reads averages and stores five-minute averages of ambient temperature, ambient pressure, filter temperature and volumetric flow rate. In addition, the microprocessor also calculates the average temperatures and pressure, total volumetric flow for the entire sample run time and the coefficient of variation of the flow rate.

Calculation

$PM_{2.5}$ was calculated based on the following formula :
$PM_{2.5}$ = (Mf – Mi) mg x 10^3 µg

where, $PM_{2.5}$ = total mass of fine particulate collected during sampling period, Mf = final mass of the conditioned filter after sample collection, Mi = initial mass of the conditioned filter before sample collection.

Appendix 3
Estimation of Carbon monoxide in Ambient Air
(BIS 5182: P10, 1999)

Principle

Carbon monoxide reduces yellow silico molybdate to lower oxides. The colour changes from yellowish green to green and finally deep blue depending on the extent of the reduction which again under identical conditions depends on concentration of carbon monoxide in air. This is estimated by indicator tube using silico molybdate method

Procedure

250 ml of the sample was drawn at the prescribed rate as per the manufacturer's instruction (40 ml / minute) through the tube by the aspirator provided. Compared the color produced with the standard colors and calculated the concentration of carbon monoxide.

Appendix 4
Estimation of Nitrogen dioxide in Ambient Air
Modified Jacobs and Hochheiser Method
(BIS 5182: P6: 2006)
(Lodge, 1988)

Estimation of Nitrogen dioxide in ambient air (Modified Jacob and Hocheiser method)
Principle

Ambient nitrogen dioxide is collected by bubbling air through a solution of sodium hydroxide and sodium arsenite. The concentration of nitrite ion (NOx) produced during sampling period is determined colorimetrically by reacting the nitrite ion with phosphoric acid, sulfanilamide and N (1-naphythyl)- ethylenediamine dihydrochloride (NEDA) and the absorbance of the highly coloured azo dye is read at 540 nm.

Reagents

1. Absorbing Reagents: 4 g NaOH and 1 g $NaAsO_2$ in 1000 ml H_2O.
2. Hydrogen peroxide: 0.024 %
3. N-(1-Naphthyl)-Ethylenediamine Di-Hydrochloride (NEDA): 1 %
4. Phosphoric Acid: 85 %
5. Sodium nitrite Solution: 1 µg NO_2 / ml
6. Sulphanilamide Solution: 20 g in 700 ml H_2O. Added 50 ml of 85 % phosphoric acid and diluted to 1000 ml.

Sampling procedure

30 ml of absorbing solution was placed in an impinger and sampled for four hours at the flow rate of 0.2 L / min. After sampling, the volume of the sample was measured before transfer to another storage bottle. 10 ml of the collected sample was pippeted into 50 ml of volumetric flask followed by the addition of 1 ml of hydrogen peroxide solution, 10 ml of sulphanilamide solution and 1.4 ml of NEDA solution, with thorough mixing after the addition of each reagent and was made upto 50 ml with distilled water. The blank was prepared in the same manner using 10 ml of unexposed absorbing reagent. It was incubated for 10 minutes and later measured and recorded the absorbance of the sample and reagent blank at 540 nm.

Calculation

The concentration of the nitrogen dioxide was calculated using the formula:
C (NO_2 µg / m^3) = (As – Ab) x CF x Vs / Va x Vt x 0.82

where, C NO_2 = Concentration of nitrogen dioxide, µg / m^3, As = Absorbance of sample, Ab = Absorbance of reagent blank, CF = Calibration factor, Va = Volume of air sampled, Vs = Volume of sample, Vt = Volume of aliquot taken for analysis, 0.82 = Sampling efficiency.

Appendix 5
Estimation of Sulphur dioxide in Ambient Air
Modified West and Gaeke Method
(BIS 5182:P2:2001)

Principle

Sulphur dioxide from air is absorbed in a solution of potassium tetrachloromercurate (TCM). A dichlorosulphitomercurate complex,which resists oxidation by the oxygen in the air is formed. The complex is made to react with para-rosaniline and formaldehydddddde to form the intensely coloured pararosaniline methylsulphonic acid. The absorbance of the solution is measured by means of a suitable spectrophotometer.

Procedure:

30 ml of absorbing solution was placed in an impinger and sampled for 4 hours at the flow rate of 1l / min. The volume of sample was measured after sampling and transferred to a sample storage bottle. The water lost by evaporation during sampling was replaced by adding distilled water up to the calibration mark of the absorber. It was then mixed thoroughly and 10 ml of the sample was pipette into 25 ml volumetric flask. To this added 1 ml of 0.6% sulphamic acid and allowed to react for 10 minutes to destroy the nitrates generated from oxides of nitrogen followed by the addition of 2 ml of 0.2% formaldehyde and 2 ml of pararosaniline solution and made up to 25 ml with distilled water. A blank was prepared using 10 ml of unexposed absorbing reagent. The absorbance of the sample was measured after 30 minutes interval of colour development and before 60 minutes at 560 nm against reagent blank. The actual concentration of the sulphite solution was determined by adding excess iodine and back titration with standard sodium thiosulfate solution. For back titration, 50 ml of the 0.01 N iodine solution was measured into each of two 500 ml iodine flasks A and B. Into flask A (blank) added 25 ml of distilled water and into flask B (sample) measured 25 ml sulphite solution. The flasks were stoppered and allowed to react for 5 minutes. To this working sulphite TCM solution, iodine was added to the flasks. These solutions were titrated against 0.01N thiosulfate until the appearance of pale yellow in the flask solution. 5 ml of starch solution was added to the flask and the titration was continued until the blue colour disappears. A graph was plotted with absorbance on y axis versus concentration on x axis. The line of best fit was drawn and the slope was determined. The reciprocal of the slope is the calibration factor.

Calculation

Concentration of sulphite solution:

C = [(V1-V2) x N x K] / V

where, C = Sulphur dioxide (SO_2) concentration, V1 = Volume of thiosulfate for blank, V2 = Volume of thiosulfate for sample, N = Normality of thiosulfate, K = 32000 (Milliequivalent weight SO_2 / µg), V = Volume of standard sulphite solution,

C (SO_2 µg / m^3) = (As – Ab) x CF x Vs / Va x Vt

where, C (SO2) = Concentration of Sulphur dioxide, As = Absorbance of sample, Ab = Absorbance of reagent blank, CF = Calibration factor, Va = Volume of air sampled, Vs = Volume of sample, Vt = Volume of aliquot taken for analysis

Appendix 6
Estimation of Ammonia
Indophenol Blue Method
(BIS 11255: P06: 1999)

Principle:

Ammonia in the atmosphere is collected by bubbling a measured volume of air through a dilute solution of sulphuric acid to form ammonium sulphate. The ammonium sulphate formed in the sample is analyzed colorimetrically by reaction with phenol and alkaline sodium hypochlorite to produce indophenol. The reaction is accelerated by the addition of sodium nitroprusside as catalyst.

Reagents:

1. Sulphuric acid (Absorbing solution): 1 N
2. Sodium nitroprusside
3. Sodium hydroxide: 6.75 M
4. Sodium hypochlorite solution
5. Phenol solution 45% v / v
6. Sodium phosphate
7. Ammonium chloride or Ammonium Sulfate
8. Hydrochloric acid
9. Ammonia stock solution (1 mg NH_3 / ml)
10. Ammonia working solution (10 µg NH_3 / ml) (Prepared fresh daily)

Procedure:

10 ml of absorbing solution was [laced in an impinge and sampled for one hour at the flow rate of 1 l / min. After sampling, measured the volume of sample and transferred contents of the sample bottle to a 25 ml glass stopper graduated cylinder. All the solutions and the sample were maintained at 25°C. To this was added 2 ml of buffer and 5 ml of working phenol solution, mixed and filled to about 22 ml followed by the addition of 2.5 ml of working hypochlorite solution and was rapidly mixed. It was then diluted to 25 ml and stored in dark for 30 minutes for colour development. The absorbance of the colour developed was measured at 630 nm against reagent blank and field blank.

Calculation:

$C(NH_3\ \mu g / m^3) = (As - Ab) \times CF / Va$

where, C NH_3 = Concentration of Ammonia in µg / m^3, As = Absorbance of sample,
Ab = Absorbance of reagent blank, CF = Calibration factor, Va = Volume of air sampled

Appendix 7
Estimation of Ozone in Ambient Air
Chemical Method
(BIS 5182:P9:1974)

Principle

Micro-amounts of ozone and the oxidants liberate iodine when absorbed in a 1% solution of potassium iodine buffered at pH 6.8. The iodine is determined spectrophotometrically by measuring the absorption of tri-iodide ion at 352 nm.

$$O_3 + 3KI + H_2O \longrightarrow KI_3 + 2KOH + O_2$$

Reagents

1. Absorbing Solution (1% Potassium iodide (KI) in 0.1 M Phosphate Buffer): 13.6 g Dihydrogen potassium phosphate (KH_2PO_4), 14.2 g Disodium Hydrogen Phosphate (Na_2HPO_4),10.0 g KI in 1L water pH 6.8
2. Stock Solution 0.025 M (Iodine) I_2 (0.05N): 16 g KI; 3.173 g of I_2 in 500 ml water; Standardized shortly before use, against 0.025 M Sodium sulphite ($Na_2S_2O_3$).

Procedure

10 ml of absorbing solution was placed in a standard impinger and sampled for one hour at the flow rate of 1l/ min. Water was added to bring the liquid volume to 10 ml. Within 30 to 60 minutes after sample collection, the absorbance was read at 352nm against a reference cuvette containing distilled water. The absorbance of the unexposed reagent was measured and it was subtracted from the value of the absorbance of the sample.

Calculation

The ozone concentration was calculated using the formula:

$C\ (O_3\ \mu g / m^3) = (As - Ab) \times CF \times 1.962 / Va$ where, C O_3 = Concentration of ammonia in µg / m^3, As = Absorbance of sample, Ab = Absorbance of reagent blank, CF = Calibration factor, Va = Volume of air sampled, 1.962 = Conversion factor.

Appendix 8
Estimation of Benzene in Ambient Air
Gas Chromatographic Method
(BIS 5182: P11: 2006)

Principle

Ambient air is sucked through charcoal tube using a low flow sampler used for collection of benzene, toluene and xylene sample in a way that results in an enrichment of the relevant substances in the activated charcoal. Resorption of the adsorbed benzene is done using carbon disulphide (CS_2). The substances desorbed in the CS_2 are analyzed by capillary gas chromatography. A flame ionization detector (FID) is used for analysis while quantification is performed using the internal / external standard.

Reagents

1. Chromatographic grade activated charcoal
2. Carbondisulphide: Chromatographic grade
3. Benzene: Chromatographic grade

Procedure

Samples were collected on glass sampling tube filled with activated charcoal at a flow rate of 100 ml / minute. Samples collected through active sampling (sorbent tubes) were extracted with 1ml of CS_2 using ultrasonication for 15 minutes to remove analyte from the sorbent material. Desorbed samples were analyzed using gas chromatography (GC) fitted with capillary column and flame ionization detector (FID). The following set of conditions were used: Gas flow: Nitrogen: 30 ml / min; (FID make up + Column); (Column flow 1 ml / minutes approximately); Hydrogen: 30 ml / min; Air :300 ml / min; Capillary column 624, Coating: Cyano propyl phenyl poly siloxane, Length x ID: 30 m **x** 0.25 mm, Film thickness: 1.4 µm; Temperature programming: Injection port: 250°C, FID: 300°C; Column / Oven: 50°C (hold for 3 min) , ramp @) 10°C / minutes to 1400°C (1 min) ramp 2 @ 20°C / minutes to 240°C (1 min); Injection volume: 5 µl, Total run time: 19.5 min, Split: 10 min; Benzene RT 6.80 min, Search window: 1.00 s, 3.00 %

Calculations

The concentration of benzene at ambient condition in µg / m^3 is calculated using the formula:

C X 101.3 (273 + T) / (273 X P)

where, C = concentration at ambient condition (µg / m^3),

T= temperature of ambient air (ºC), P = atmospheric pressure (kPA).

The concentration at ambient condition is calculated by the formula:

(C X V1 X 1000) / V2 X V3

where, C = amount of compound found in the injection sample volume obtained from standard curve (µg / µl), V2 = total volume of sample extract injected into GC (µl) which in turn is calculated by the formula = S X t / 106

where S = sampling flow rate (ml / min). t = sampling time (min),

V3 = Volume of air drawn through the tube (m^3)

Appendix 9
Estimation of Benz(a)pyrene in Ambient Air
Solvent Extraction and Gas Chromatography Analysis
(BIS 5182: P12: 2004)

Principle

Particulate phase PAHs are collected in ambient air and individual PAH compounds are determined using capillary gas chromatograph equipped with flame ionization detector. It is a high volume (1.2 m^3 / min) sampling method capable of detecting very low concentrations of PAH.

Procedure

Sampling was done for 8 hours using PM_{10} high volume sampler using EPM-2000 glass fibre, at a flow rate of 1m^3 / minutes per minute. After sampling, filters were kept in the controlled laboratory conditions (20 - 25°C) Filter papers were cut into strips using scissors and transferred to 250 ml beaker. Added 50 ml of toluene (GC / HPLC grade) and extracted using ultra sonic bath for about 30 minutes. The procedure was repeated twice (50ml x 2 times) for complete extraction.

The extracted samples were filtered with Whatman filter paper no.41 containing 2 g of anhydrous sodium sulphate. After filtration, the filtrate was concentrated using Rotary vacuum evaporator to 2ml final volume. Passed 2 ml of concentrated sample through silica gel column (pre conditioned, 60-80 mesh and 200-250 mm×10 mm with Teflon stopcock) to clean up the impurities. After cleaning, 5 ml of cyclohexane was added and collected the eluting fluid in 25 ml beaker. The process was repeated thrice and collected it in the same beaker. The cleaned up extract / filtrate (approximately 17 ml) is further concentrated using rotary evaporator and it is evaporated to nearly dryness with nitrogen. The dried sample was re-dissolved in 1ml of toluene and transferred into 4 or 5 ml amber vials for final analysis. GC Conditions: Injector: 300°C; FID Temp: 320°C; Column: Ultra - 2 (25m Length, 320µm diameter, 0.17µ) or equivalent; Oven: 120°C →2 minutes hold → 7°C / minutes → 300°C → 10 minutes hold; Run Time: 37.71 minutes; Carrier gas flow (N_2): 0.50 ml / min; Gases for FID Flame: H_2 flow: 40 ml / min; Zero grade air flow: 400 ml / min; After internal and external calibration, 2 µl of sample from the amber vial using standard gas tight syringe was injected in the Capillary GC-FID instrument for analysis. Recorded the resulting concentration of each PAH compound including B(a)P.

Calculations

The concentration in ng / µl of each identified analyte or B(a)P was calculated in the sample extract (Cs) as follows:

$V = Q \times T$

where, Q =Average flow rate of sampling m^3 / min,

T = sampling time, V = total sample volume at ambient conditions,

Concentration of B(a)P : C (ng / m^3) = Cs * Ve / Vi * Vs where,

Cs: Concentration of Benzo (a) pyrene in ng / µl in the sample extract recorded by GC.

Ve: Final volume of extract in µl (i.e 1000),

Vi : Injection Volume (i.e 1µl),

Vs : Volume of air sample.

Appendix 10
Estimation of Lead, Arsenic and Nickel in Ambient Air
Atomic Absorption Spectroscopy
(BIS 5182: P22: 2004)
(USEPA – IO 3.2)
(APHA, 1998)

The Atomic Absorption Spectroscopy (AAS) technique makes use of absorption spectrometry to assess the concentration of an analyte in the sample. The method is based on active sampling using PM10 High Volume Sampler and then sample analysis is done by atomic absorption spectrophotometer (Lodge, 1988).

Principle

A light beam containing the corresponding wavelength of the energy required to raise the atoms of the analyte from the ground state to the excited state is directed through the furnace. This wavelength is observed by a monochromator and a detector that measure the amount of light absorbed by the element, hence the number of atoms in the ground state in the furnace. A hollow cathode lamp for the element being determined provides a source of that metal's particular absorption wavelength.

The sample of air is drawn through a sampling train consisting of a 0.45 pm membrane filter or its equivalent and then through a special sampling tube containing activated carbon. A sample shall be collected at a flow rate of 1 to 1.5 litres per minute continuously for 8 h. A plastic body or glass tube type rotameter (0.5 cpm range) may be used either on or off line for measuring flow and its variation on filter. The particulate sample is digested with nitric acid and perchloric acid and dissolved lead, nickel and arsenic are determined by Atomic Absorption Spectroscopy.

Reagents

1. Filter paper: EPM 2000 or equivalent, 20.3 X 25.4 cm (8 X 10 in)
2. Hydrochloric acid (HCl) concentrated
3. Nitric acid (HNO_3) concentrated
4. Sulphuric Acid (H_2SO_4) concentrated
5. Metal standard solutions

6. Sodium borohydride
7. Potassium iodide

Procedure

Sampling Procedure and Extraction

The inlet of the PM_{10} high volume sampler was prepared with 47 mm PFTE filter with the rough side of the filter facing upwards. Gently the inlet was lowered. The reading of the elapsed time meter was recorded. Air sample was drawn through the sampler in to the filter. The specified length of sampling used for this analysis is 8 hours at a flow rate of 1 L / minute. After the required time of sampling, the flow meter was recorded and the filter was taken out from the sampler and put in a container and were stored at 30°C until extraction and further analysis. The collected sample on glass fibre filters was extracted by hot plate procedure by acid digestion (3% Nitric acid and 8% Hydrochloric acid). The beaker was placed on the hotplate, contained in a fume hood, and refluxed gently while covered with a watch glass for 30 min. The beaker was removed from the hot-plate and allowed to cool. The beaker walls were rinsed and washed with distilled water. Approximately 10 ml reagent water was added to the remaining filter material in the beaker and allowed to stand for at least 30 min. The extraction fluid was transferred into a 100 ml volumetric flask. The beaker was rinsed with distilled water to remove any remaining solid material and added the rinses to the flask. This was then made up to the mark by dilution with deionised water and was shaken well. The final extraction solution concentration is 3 % HNO_3 and 8% HCl. The filtered sample is now ready for analysis

Analysis of samples

A series of metal standards of interest were run and constructed a calibration curve followed by the aspiration of the samples. For Lead (Pb) and Nickel (Ni), the wavelength required for analysis is 217nm and 232nm respectively, whereas in case of Arsenic (As), the Vapour Generator Assembly (VGA) was attached with flame and the wavelength required for analysis is 193.7 nm.

Calibration

The standard solutions were prepared from the stock solutions. At least three standards were selected to cover linear range as recommended by method. Injected the standards into the furnace and recorded the absorbance. Prepared the calibration graph by plotting absorbance and concentration in µg/ml.

Calculations

The concentration of metal is calculated using the following formula :

$C = (Ms – Mb) \times Vs \times Fa / V \times Ft$

where, C = concentration, Ms = metal concentration, Mb = blank concentration,
Vs = total volume of extraction, Fa = total area of exposed filter,
V = Volume of air sampled, Ft = Area of filter taken for digestion where sample air volume is

$V = (Q) (t)$

where, V = volume of air, Q = average sampling rate, t = time in minutes.

Appendix 11
Physical Characterization of Toner by Scanning Electron Microscopy Energy-Dispersive X-ray Spectroscopy SEM EDAX (Egan *et al*., 2003)

Principle:

Scanning Electron Microscopy (SEM) is a commonly used technique for imaging materials on the micro to nanometer scale. A Scanning Electron Microscope produces images by probing the sample with a beam of electrons which is focused into a spot on the sample surface. The beam is raster-scanned across the specimen. As the beam interacts with the specimen, various processes occur, including generation of secondary electrons, X-rays and re-exiting, or back scattering of some of the electrons that entered the sample. These backscattered or the secondary electrons can be used to construct an image of the surface topography; a SEM image is a plot of the relevant signal at the x,y position that the signal was generated. The X-rays generated as a result of the interaction of the electrons with the sample contain can be detected and enable an analytical technique called Energy-dispersive X-ray Spectroscopy (EDS or EDX)

which is used for elemental analysis and chemical characterisation of the specimen; each element in the specimen has a unique atomic structure which results in a unique set of peaks in the EDS spectrum

Procedure:

The toner sample was attached to double-sided tape applied to a carbon planchet and made conductive by coating with carbon by vacuum evaporation. Carbon coating was mandated by the sample characteristics (conductivity and size), operating environment (pressure), and analytical requirements (beam voltage and current). Elemental analysis of toner samples was performed using a JSM-6390 (JEOL, Japan)), with a EDXA attachment (EDX Oxford Instrument, INCA PentaFET X3) For X-ray analysis, spectra were obtained using 25 KeV beam voltage, ca. 1.3 nA beam current (adjusted to yield a dead time of 30%), and 100 s live time. The instrument design employed a 39 mm working distance to provide optimal sample/detector geometry. The scan was rastered over the surface of an area of intact toner. For each sample, relative abundances of elements were calculated by dividing the net peak counts (above background) of the principal (strongest) peak for each element by the sum of counts from all elements present

Appendix 12
Screening for Volatile Organic Compounds (VOCs) by Head Space Gas Chromatography - Mass Spectrometry (GC- MS) (USEPA, 8260C)

Sample preparation

1 g of toner was placed in a capped 20 ml sample vial containing air and heated to 185 C for 20 min and at the end of that time, a 300 ul sample of the headspace gas was withdrawn and used for injection into GC/MS column. The peaks identified were matched against computer library of mass spectra . screening procedure matched

Procedure:

Analysis of toner samples was performed using a DB-5MS capillary column (30 m×0.25 mm, 0.25 µm, Agilent technologies Inc., Santa Clara, CA, USA) in a GC/MS (5975C, Agilent technologies Inc). 1 ul sample was injected into the column and ran using split mode (split ratio= 10:1). The helium carrier gas was programmed to maintain a constant flow rate of 1.5 mL/min. Oven was initially maintained at initital isothermal period of 3 min at 70 °C, then finally raised to 300°C at the rate of 10°C /min for 9.0 minutes for a total of 35 minutes. Mass analysis detector was used with the following conditions (Ion source temperature 230 C, Interface temperature 240 C, Scan range 400 – 700 m/z, solvent cut time 3 minutes, MS start time 3 min – end time 35 minutes, Ionisation at -70 eV and scan speed of 2000. The peaks obtained were matched against computer library of mass spectra (NIST 11), screening procedure matched

Appendix 13
Informed Consent Form

AVINASHILINGAM DEEMED UNIVERSITY FOR WOMEN
COIMBATORE – 641 043, TAMILNADU, INDIA

The title of our research project is "Air quality monitoring and health surveillance of the workers in photocopier units".

Principal Investigator	***:***	***Dr.G.P.Jeyanthi***
Organisation	***:***	***Department of Biochemistry, Biotechnology & Bioinformatics, Avinashilingam Deemed University for Women, Coimbatore - 641 043, Tamilnadu, India.***
Sponsor	***:***	***Indian Council of Medical Research Government of India, New Delhi***
Name of Proposal	***:***	***Air quality monitoring and health surveillance of the workers in photocopier units***

This Informed Consent Form has two parts:

I. **Information Sheet (to share information about the research with you)**
II. **Certificate of Consent (for signatures if you agree to take part)**

You will be given a copy of the full Informed Consent form

PART I: Information Sheet

Introduction

I am G.P.Jeyanthi, working for Avinashilingam Deemed University for Women, Coimbatore. We are doing research on health status of workers in photocopier units. I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask about them to me, the study doctor or the staff.

Purpose of the research

Workers in photocopier/ xerox units are inhaling a lot of gases emitted from the machines during the course of their work. The toners also contain a mixture of chemicals which may be harmful to human health. This research is being done to find out if inhalation of the toxic gases emitted from Xerox machines is harmful to human health.

Type of intervention

This research will involve assessment of lung function by spirometry, blood sample by a single prick from your arm followed by collection of urine by non invasive method to determine selected blood and urine biochemical parameters.

Participant selection

We are inviting all adults in the age group of 21-50 years who have been working in photocopier units for a period of at least 2 years in and around Coimbatore, to participate in the research on health status of workers in photocopier units.

Voluntary Participation

Your participation in this research is entirely voluntary. You may withdraw from the study at any point of time if you are not interested in it. It is your choice whether to participate or not.

Procedures and Protocol

A. Sample Collection Procedures

We will be testing your lung function by using a spirometer. In this test, we will ask you to blast air into a mouthpiece connected to a machine for a period of about 15 minutes and get the result printed from the machine. We will take one table spoon of blood only once from your arm using a syringe and needle. For collection of exhaled breath condensate, we will ask you to breathe through a mouth piece for a period of 10 minutes. We will also ask you to provide 20 ml of your urine sample. At the end of the research, leftover sample will be destroyed by incineration.

B. Description of the Process

- *At first, we will ask you certain questions about your personal, professional and clinical history to fill in an interview schedule.*
- *Following this lung function test is performed using a spirometer for which you will have to blow air through a mouthpiece connected to the instrument (for a period of 15 minutes) until satisfactory results are got.*
- *Next, about 5.0 ml of blood, equal to about a tablespoon, will be taken from your arm with a syringe by a single prick. This blood will be tested for the presence of substances that will help your body to defend against toxic chemicals.*
- *Next, we will ask you to give about 20 ml of urine sample*

C. Duration

The research takes place over a period of three years. During this period you will be asked to give your all samples once.

Benefits

There are no benefits for you from this study but your participation and co-operation is likely to help us to find an answer for our research question. Bu the results of this study will be a useful aid for the future, to tell you whether working in Xerox units causes any health problems or it needs more ventilation or working hours per day needs to be reduced or you have to be given any compulsory week end holidays.

Risks and side effects

There are no potential risks and side effects in participating in this study.

Reimbursements

You will not be given any other money or gifts to take part in this research study.

Confidentiality

We will not reveal or disclose or share the identity of those participating in the research. It will not be given to anyone or for any publications. All the details will be kept confidential.

Sharing the Results

We will give you a copy of the informed consent, results of the lung function test and initial results of the blood test as part of the study

Right to Refuse or Withdraw

It is your choice to either participate or refuse to participate. You have all the rights to withdraw at any point of time during the study.

Whom to Contact

If you have any questions you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact any of the following:

Dr.G.P.Jeyanthi, Professor,
Department of Biochemistry, Biotechnology and Bioinformatics,
Avinashilingam Deemed University for Women,
Coimbatore – 641 043, Ph: 98650 05330,
Email: jeyanthigp@yahoo.co.in

This proposal has been reviewed and approved by Institutional Ethics Committee, Avinashilingam Deemed University for Women, Coimbatore, which is a committee whose task it is to make sure that research participants are protected from harm. If you wish to find about more about the Institutional Review Board, contact

Dr. Premakumari, Convener, Institutional Ethics Committee,
Avinashilingam Deemed University for Women,
Coimbatore – 641 043.Ph: 0422-2440241

It has also been reviewed by the ***Ethics Review Committee of the Indian Council of Medical Research, Government of India*** *which is funding the study.*

- *Do you know that you do not have to take part in this study if you do not wish to?*
- *Do you know that you can say No if you wish to? Do you know that you can ask me questions later, if you wish to?*
- *Do you know that I have given the contact details of the person who can give you more information about the study?*

*You can ask me any questions about any part of the research study, if you wish to. Do you have any more questions**?***

PART II: Certificate of Consent

I have read the foregoing information, or it has been read to me. I consent voluntarily to participate as a participant in this research.

Print Name of Participant_____

Signature of Participant _____

Date _____

If illiterate

I have witnessed the accurate reading of the objectives, study protocol and informed consent form to the participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given voluntary consent.

Print name of witness_____ and Thumb print of participant

Signature of witness _____

Date _____

Statement by Researcher / Person taking Consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. ***Lung function assessment by Spirometry***
2. ***Collection of blood sample (5 ml)***
3. ***Collection of exhaled breath condensate (1 ml)***
4. ***Collection of urine sample (25 ml)***

I confirm that the individual has not been coerced to give consent, and the consent has been given voluntarily after informing the study objectives and the protocol .

A copy of this ICF has been provided to the participant.

Print Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

Appendix 14
Interview Schedule

PERSONAL DETAILS				
Name				
Age				
Residential address ☎				
Office address ☎				
Occupation				
Educational qualification				
Family income				
Married	Yes	No		
Fuel used for cooking	LPG	Kerosene	Firewood	Others

Do you smoke	Yes	No
Smoker	**Exsmoker**	**Nonsmoker**
No. of cigarettes / day		
No.of years of smoking		
Passive smoking	Yes	No
Any other addictions	Yes	No
If yes, specify		
Professional background		
No. of years in photocopier units		
No. of working hours / day		
No. of working days / week		
Photocopier make / model		
How often serviced		
Toner brand		

HEALTH SURVEY

Do you suffer from any of the following?	**Frequency**	**Duration**	**Treatment**
Nose / throat irritation			
Dry skin / Redness of skin			
Tiredness			
Headache			
Throat pain			
Nausea			
Do you suffer from any of the following?	**Frequency**	**Duration**	**Treatment**
Dizziness			
Nausea			
Blocked nose, runny nose / sneezing fits			

Eye problems / irritations		
Persistent cough		
Persistent phlegm		
Asthma		
Wheezing		
Tightness in the chest		
Allergies		
Shortness of breath		
Do you regularly suffer from respiratory problems?	Yes	No
Frequency of attacks	**Seasonal**	**Throughout the year**
If seasonal, during which season		
Do you have any other symptoms which are not covered by these questions?		
Do these symptoms trouble you while you are working? **Yes No**		
Are the symptoms you have indicated possibly due to your work? **Yes No**		
If so, which are these symptoms caused or made worse by your work?		

CLINICAL HISTORY

Have you been treated for any of the following diseases? **Yes No**	
Diabetes	
High blood pressure	
Cardiovascular disease	
Nervous tension	
Epilepsy	

Insomnia		
Skin diseases		
Bronchitis (or asthma)		
Lifestyle		
Diet Vegetarian Non Vegetarian		
Do you include fresh foods in your diet?	**Yes**	**No**
Do you, in your leisure time, regularly play sports / do exercise?	**Yes**	**No**
Do you limit your intake of fats in your diet?	**Yes**	**No**
Do you eat leisurely?	**Yes**	**No**
Do you drink alcohol?	**Yes**	**No**
How often and how much do you have?		
Awareness about emissions from photocopiers	**Yes**	**No**
Do you know about the emissions from the photocopier?	**Yes**	**No**
What have you done to protect yourself from the emissions from photocopier?		

Appendix 15
Estimation of Plasma Glucose
Glucose oxidase Kit Method (Agappe Diagnostics, India)
(Trinder, 1969)

Principle

Glucose was estimated based on the following enzymatic reactions:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} H_2O_2 + \text{Gluconic acid}$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminophenazone} \xrightarrow{\text{POD}} \text{Quinonimine} + 4\ H_2O$$

GOD – Glucose oxidase; POD – peroxidase

Reagents

1. Glucose reagent 1: Tris buffer (pH 7.4) – 92 mM; Phenol - 0.3 mM; Glucose oxidase - 15000 U / L; 4 Aminophenazone- 2.6 Mm

2. Glucose standard : 100 mg / dl

Procedure

Each 10 µl aliquot of standard and serum sample were mixed with 1 ml of glucose reagent and incubated for 10 minutes at room temperature. Absorbance of the colour developed was measured spectrophotometrically at 505 nm against reagent blank.

Calculation

Sample concentration = [Abs ($_{sample}$) / Abs ($_{standard}$)] x standard concentration

Appendix 16
Estimation of Serum Total Protein
Biuret Method (CPC Diagnostics, India)
(Gornall *et al.,* 1949)

Principle

Protein in serum forms a blue coloured complex on reaction with cupric ions in an alkaline solution. The intensity of the colour is proportional to the amount of protein present when compared to a solution with known protein concentration.

Reagents

1. Protein reagent: Copper (II) ions - 6mmol / L; NaOH-1.15mol / L
2. Standard albumin: 50.7 g / l

Procedure

10 µl aliquots of standard and serum sample were mixed with 1 ml of assay reagent and incubated for 1 minute at room temperature. Absorbance of the colour developed was measured spectrophotometrically at 630 nm against water blank.

Calculation

Sample concentration = [Abs (sample) / Abs (standard)] x standard concentration

Appendix 17
Estimation of Serum Albumin
Bromocresol Green Method (CPC Diagnostics, India)
(Doumas *et al.,* 1971)

Principle

Serum albumin binds selectively to the dye bromocresol green in acid medium. The increase in absorbance of the resulting albumin-dye complex, read at 630 nm, is proportional to albumin concentration.

Reagents

1. Albumin reagent: Acetate buffer-100mmol / L; Bromocresol green: 0.27mmol / l; pH 4.1

2. Standard bovine albumin: 6.5 g / dl.

Procedure

To 10 µl aliquots of standard and serum sample, added 1 ml of assay reagent. Mixed thoroughly and incubated for 1minute at room temperature. Absorbance of the colour developed was measured spectrophotometrically at 630 nm against water blank.

Calculation

Sample concentration = [Abs ($_{sample}$) / Abs ($_{standard}$)] x standard concentration

Appendix 18
Estimation of Lipid peroxides in Serum
Thiobarbituric Acid Reactive Substances (TBARS) Assay
Colorimetric Method
(Jentzsch *et al.,* 1996)

Principle

One molecule of malondialdehyde reacts with two molecules of thiobarbituric acid to produce a pink pigment with absorption at 535 nm. Amplification of peroxidation during the assay is prevented by the addition of the chain breaking antioxidant butylated hydroxy toluene (BHT).

Reagents

1. Standard malondialdehyde (MDA): 0.2 – 4 µM 1,1,3,3 tetramethoxy propane
2. Ortho phosphoric acid: 0.2 M
3. Butylated Hydroxy Toluene (BHT): 2 mM
4. Thiobarbituric acid reagent (TBA): 0.11 M in 0.1M NaOH
5. n butanol

Procedure

To each of 400 µl aliquots of serum, water blank, dilutions of different MDA standard, added 400 µl aliquots of orthophosphoric acid reagent, 50 µl aliquots of BHT reagent and 50 µl aliquots of TBA. Mixed well and incubated at 90°C for 45 minutes in a water bath. The reactions were stopped by transfer of the tubes to ice bath, followed by the addition of 1ml of n-butanol. The butanol phase was transferred to fresh tubes after vortex for 20 seconds. Read the absorbance of organic phase at 535 nm and 572 nm (for base line correction). MDA equivalents were calculated using the difference in absorption at two wavelengths. TBARS levels were calculated by constructing a linear graph of the standard concentrations.

Appendix 19
Estimation of Plasma Free 8-Isoprostane
Competitive Enzyme Immuno Assay (EIA) Kit Method (Cayman Chemical, USA)
(Pradelles *et al.,* 1985)

Principle

This assay is based on the competition between free 8-isoprostane and an 8-isoprostane-acetylcholinesterase (AChE) conjugate (8-Isoprostane tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. The concentration of the 8-Isoprostane tracer is held constant while the concentration of free 8-isoprostane varies, the amount of 8-Isoprostane tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of free 8-isoprostane in the well. This rabbit antiserum 8- isoprostane complex binds to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The enzymatic reaction product has a distinct yellow colour. The intensity of this color, determined spectrophotometrically at 412 nm, is proportional to the amount of 8-Isoprostane tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane.

Description of each well in the ELISA plate of Free-8 Isoprostane kit

Blank well denotes absorbance of Ellman's reagent which is subtracted from the absorbance readings of all the other wells.

Total Activity well denotes total enzymatic activity of the AChE-linked tracer.

NSB (Non-Specific Binding) well denotes non-immunological binding of the tracer to the well.

B_0 (Maximum Binding) denotes maximum amount of the tracer that the antibody can bind in the absence of free analyte.

% B / B_0 (% Bound / Maximum Bound) well denotes ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Reagents

1. Enzyme Immuno Assay buffer (EIA)
2. Wash Buffer
3. Plasma: Plasma samples for free 8-Isoprostane were stored at - 80°C in presence of 0.005% BHT till the time of assay
4. 8-Isoprostane EIA Standard: 0.8 - 500 pg / ml.
5. 8-Isoprostane Acetylcholine tracer (Ache)
6. 8-Isoprostane antiserum
7. Ellman's reagent

Procedure

Into the ELISA plate added 100 µl and 50 µl aliquots of EIA buffer to non specific binding wells and maximum binding wells correspondingly. Subsequently added 50 µl diluted standards in the following concentration ranging from 500, 200, 80, 32, 12.8, 5.1, 2.0,0.8 pg / ml into the standard wells and plasma sample to the remaining wells followed by the addition of 50 µl aliquots of Ache tracer to all wells except total activity and blank wells. Subsequently, added 50 µl of EIA antiserum to all wells except total activity, blank and non specific binding wells. Covered the plate with plastic film and incubated at 4°C for 18 hours. The wells were emptied and rinsed five times with wash buffer. Later, added 200 µl of Ellman's reagent to all wells. Finally added 5 µl tracer specifically to total activity wells. Then the plate was incubated at room temperature in dark for 2 hours with continuous shaking on orbital shaker. Absorbance was read at 415 nm.

Calculation

The binding in each well was calculated by subtracting the absorbance of NSB wells from the absorbance in each well. The %B / B_0 for the standard and sample wells was calculated from the above calculated values. A four parametric logistic fit graph of %B / B_0 versus log concentration was plotted to calculate the plasma levels of free 8-Isoprostane.

Appendix 20
Estimation of Plasma Trolox Equivalent Antioxidant Capacity Colorimetric Kit Method (Cayman chemical, USA) (Miller *et al.,* 1993)

Principle

Antioxidants inhibit the oxidation of ABTS* (2, 2'-Azino-di-[3-ethyl benzthiazoline sulphonate]) to ABTS**+ by metmyoglobin. The amount of ABTS**+ produced is monitored by reading the absorbance at 750 nm. The concentration of antioxidant is proportional to the degree of suppression at 750 nm. The antioxidant's capacity to prevent ABTS* oxidation is compared with that of Trolox, a water soluble tocopherol analogue.

Reagents

1. Assay buffer: 0.5 mM Potassium phosphate; 0.9 % NaCl; 0.1 % glucose; pH 7.4
2. Chromogen: ABTS*
3. Metmyoglobin
4. Trolox standard: Trolox standards of 0.045 - 0.33 mM
5. Hydrogen peroxide: 441 µM H_2O_2
6. Plasma sample dilution: 1:25.

Procedure

Into the micotitre plate, added 10 µl aliquots of diluted Trolox standard and 10 µl aliquots of plasma into standard and sample wells correspondingly followed by the addition of 10 µl aliquots of metmyoglobin, 150 µl aliquots of chromogen and 40 µl aliquots of hydrogen peroxide solution to all the wells. Covered the plate and incubated on a shaker for 5 minutes at room temperature. Removed the cover and read the absorbance at 750 nm. Constructed a linear standard curve using MPM3 microplate reader software and calculated the Trolox equivalent antioxidant levels from the standard curve.

Appendix 21
Estimation of Serum Ferric Reducing Antioxidant Capacity Colorimetric Method (Benzie and Strain, 1996)

Principle

The reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form at a lower pH of pH 3.6 develops an intense blue colour which is monitored by measuring the change in absorption at 593 nm.

Reagents

1. FRAC Reagent: Acetate buffer 300 mM pH 3.6; TPTZ (2, 4, 6 - tripyridyl-S- triazine); 10 mM TPTZ in 40mM HCl; $FeCl_3$. $6H_2O$: 20 mM; Working standard was prepared by mixing acetate buffer, TPTZ and $FeCl_3$ in the ratio of 10:1:1 at the time of use.
2. Standard: Ascorbic Acid: 1000 µM

Procedure

100 µl aliquot of water was added to blank tubes. 100 µl of each of diluted standard and sample were added to the appropriate wells. 3 ml of working FRAC reagent was added to all the tubes. Absorbance was measured at 590 nm immediately after vortex. All tubes were incubated at 37°C for 4 minutes. Mixed well and measured the absorbance again at 590 nm.

Calculations

FRAC value of Sample (µM) = (Change in absorbance of sample from 0 to 4 minute / Change in absorbance of standard from 0 to 4 minute) X FRAC value of standard (1000 µM)

Appendix 22
Estimation of Plasma Clara Cell Protein
Sandwich ELISA Kit Method (USCN, China)
(Dierynck *et al.,* 1995)

Principle

This assay is based on the sandwich formation between the well coated CC16 antibody, sample Clara Cell protein and specific biotin conjugated CC16 antibody. The microtitre plate is pre-coated with an antibody specific to Clara Cell protein (CC16). Standards or samples are added to the appropriate wells with a biotin-conjugated antibody preparation specific for CC16. Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then TMB substrate solution is added. The wells that contain CC16, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450nm. The concentration of CC16 in the samples is directly proportional to the change in colour intensity and is obtained from the standard plot.

Reagents

1. Standard Clara Cell protein: 62.5 – 4000 pg / ml
2. Detection reagent A: Biotin conjugated antibody
3. Detection reagent B: Avidin conjugated horseradish peroxidase
4. Wash buffer
5. Substrate solution: 3,3',5,5' – tetramethyl benzidine (TMB)
6. Sulphuric acid
7. Plasma: samples were diluted 50 fold in PBS before addition to wells.

Procedure

Into the ELISA plate, added 100 µl aliquots each of seven dilutions of standard, blank and samples into the appropriate wells followed by incubation, for 2 hours at 37°C after covering the plate. Later, all the wells were decanted. Subsequently, added 100 µl of detection reagent A to all the wells followed by incubation for 1 hour at 37°C after covering with the plate sealer. Aspirated the solution from each well and washed thrice with 350 µl of wash solution. After the last wash, decanted the plate and blotted it against absorbent paper. Subsequently, 100 µl aliquots of detection reagent B was added to each well followed by Incubation for 30 minutes at 37°C after covering with plate sealer. The wash process was repeated 5 times. Finally, added 90 µl of TMB substrate solution to each well. The plate was covered and incubated for 15 – 25 minutes at 37°C in dark. 50 µl of sulphuric acid stop solution was added to each well to stop the reaction. Read the absorbance at 450 nm immediately. Created a log-log standard graph using MPM3 software and extrapolated the results for plasma samples.

Appendix 23
Estimation of Plasma Leukotriene B_4
Competitive EIA Kit (Cayman Chemical, USA)
(Pradelles *et al.,* 1985)

Principle

This assay is based on the competition between leukotriene B_4 (LTB_4) and an LTB_4-acetylcholinesterase (AChE) conjugate (LTB_4 tracer) for a limited number of LTB_4 antiserum. The concentration of the LTB_4 tracer is held constant while the concentration of LTB_4 varies, the amount of LTB_4 tracer that is able to bind to the LTB_4 antiserum will be inversely proportional to the concentration of LTB_4 in the well. This antibody- LTB_4 complex binds to a mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of LTB_4 tracer bound to the well, which is inversely proportional to the amount of

free LTB_4 present in the well.

Description of each well in the ELISA plate of Free-8 Isoprostane kit

Blank well denotes absorbance of Ellman's reagent which is subtracted from the absorbance readings of all the other wells.

Total Activity well denotes total enzymatic activity of the AChE (Acetyl Choline Esterase)-linked tracer.

NSB (Non-Specific Binding) well denotes non-immunological binding of the tracer to the well.

B_0 (Maximum Binding) denotes maximum amount of the tracer that the antibody can bind in the absence of free analyte.

% B / B_0 (% Bound / Maximum Bound) well denotes ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Reagents

1. EIA buffer
2. Wash buffer
3. Plasma: Plasma samples for LTB_4 were stored at - 80°C in the presence of 10 µM indomethacin till the time of assay
4. LTB_4 EIA Standard: 8 standards were prepared in the range of 3.9 - 500 pg / ml.
5. LTB_4 tracer
6. LTB_4 antiserum
7. Ellman's reagent

Procedure

Into the ELISA plate, added 100 µl and 50 µl aliquots of EIA buffer to non specific binding wells and maximum binding wells correspondingly. Subsequently added 50 µl diluted standards in the following concentration ranging from 500, 250, 125, 62.5, 31.3, 7.8, 3.9 pg / ml into the standard wells and plasma sample to the remaining wells followed by the addition of 50 µl aliquots of Ache tracer to all wells except total activity and blank wells. Subsequently, added 50 µl of EIA antiserum to all wells except total activity, blank and non specific binding wells. Covered the plate with plastic film and incubated at 4°C for 18 hours. The wells were emptied and rinsed five times with wash buffer. Later, added 200 µl of Ellman's reagent to all wells. Finally added 5 µl tracer specifically to total activity wells. Then the plate was incubated at room temperature in dark for 2 hours with continuous shaking on orbital shaker. Absorbance was read at 415 nm.

Calculation

The binding in each well was calculated by subtracting the absorbance of NSB wells from the absorbance in each well. The %B / B_0 for the standard and sample wells was calculated from the above calculated values. A four parametric logistic fit graph of %B / B_0 versus log concentration was plotted to calculate the plasma levels of Leukotriene B_4

Appendix 24
Estimation of Plasma Interleukin 6 (IL-6)
Sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) Kit Method
(Koma Biotech, Korea)
(Baroja *et al.,* 1988)

Principle

This assay is based on the sandwich formation between the well coated IL- 6 antibody, sample IL- 6 and specific biotin conjugated IL - 6 antibody. In the ELISA plate, wells are coated with antibody to interleukin 6 (IL- 6). On addition of the sample, the IL-6 in the sample binds to the coated antibody in the well. On further addition of Streptavidin-HRP conjugate detection antibody, it binds to the bound IL-6 in the well. A sandwich formation between IL-6 Antibody – Sample IL 6 – Streptavidin-HRP-conjugate detection antibody complex is estimated colorimetrically at 450 nm by the addition of substrate to Horse Radish Peroxidase conjugate. The intensity of the colour is directly proportional to the concentration of IL-8.

Reagents

1. Detection antibody:
   Biotinylated antigen-affinity purified Goat anti-Human IL-6 (0.25 µg / ml)
2. Standard protein: Recombinant Human IL-6 (1 µg / ml)
3. Colour development enzyme: Streptavidin – Horse Radish Peroxidase (HRP) conjugate
4. Assay Diluent: 0.1% BSA in PBS
5. Colour development reagent A: TMB solution (3,3',5,5' – tetramethyl benzidine)
6. Colour development reagent B: Substrate Solution A: H_2O_2
7. Stop solution: 2M Sulphuric acid (H_2SO4)
8. Phosphate Buffered Saline (PBS)
9. Wash solution: 1 ml Tween-20 in 1l PBS

Procedure

Into the microtitre plate, added 200 µl of wash solution to each well followed by aspiration and subsequently, repeated the washing and decanting procedure thrice using 300 µl of wash solution per well. In the final wash of decant, the plate was blotted onto paper towel to remove residual wash solution. After the washing procedure, added 100 µl of standard in the concentration range of 2000 – 31.25 pg /ml or sample to each well in duplicates. Covered and incubated the plate at room temperature for 2 hours. Aspirated the wells to remove liquid and washed the plate 4 times as before. This was followed by the addition of 100 µl of the diluted detection antibody to each well. Covered and incubated at room temperature for 2 hours. The aspiration and wash procedure were repeated 4 times. Subsequently, added 100 µl of the diluted colour development enzyme per well. Covered and incubated for 30 minutes at room temperature. The aspiration and wash procedure were repeated 4 times. To initiate the colour reaction, added 100 µl of colour development solution to each well followed by incubation at room temperature for 20 minutes. To stop the colour reaction, added 100 µl of the stop solution to each well. Read the absorbance at 450 nm. A semi log graph was constructed in MPM 3 software and the levels of IL-6 in the plasma samples were calculated.

Appendix 25
Estimation of Plasma Interleukin 8
Sandwich ELISA Kit Method (Koma Biotech, Korea)
(Sticherling *et al.,* 1989)

Principle

This assay is based on the sandwich formation between the well coated IL-8 antibody, sample IL-6 and specific biotin conjugated IL -6 antibody. In the ELISA plate, wells are coated with antibody to interleukin 8 (IL-8). On addition of the sample, the interleukin 8 in the sample binds to the coated antibody in the well. On further addition of Streptavidin-HRP conjugate detection antibody, it binds to the bound IL-8 in the well. A sandwich formation between IL-8 Antiobody – Sample IL 8 -Streptavidin-HRP-conjugate detection antibody complex is estimated olorimetrically at 450 nm by the addition of substrate to Horse Radish Peroxidase conjugate. The intensity of the colour is directly proportional to the concentration of IL-8.

Reagents

1. Detection antibody:
   Biotinylated antigen-affinity purified Goat anti-human IL-8 (0.25 µg/ml)
2. Standard Protein: Recombinant Human IL-8 (1 µg / ml)
3. Colour Development Enzyme: Streptavidin-HRP conjugate
4. Assay Diluent: 0.1% BSA in PBS
5. Colour development reagent A: TMB solution (3,3',5,5' – tetramethyl benzidine)
6. Colour development reagent B: Substrate Solution A: H2O2
7. Stop solution: 2M Sulphuric acid (H_2SO4)
8. Phosphate Buffered Saline (PBS)
9. Wash solution: 1 ml Tween-20 in 1l PBS

Procedure

Into the microtitre plate, added 200 µl of wash solution to each well followed by aspiration and subsequently, repeated the washing and decanting procedure thrice using 300 µl

of wash solution per well. In the final wash of decant, the plate was blotted onto paper towel to remove residual wash solution. After the washing procedure, added 100 µl of standard in the concentration range of (2000 – 7.8125 pg /ml) or sample to each well in duplicates. Covered and incubated the plate at room temperature for 2 hours. Aspirated the wells to remove liquid and washed the plate 4 times as before. This was followed by the addition of 100 µl of the diluted detection antibody to each well. Covered and incubated at room temperature for 2 hours. The aspiration and wash procedure were repeated 4 times. Subsequently, added 100 µl of the diluted colour development enzyme per well. Covered and incubated for 30 minutes at room temperature. The aspiration and wash procedure were repeated 4 times. To initiate, the colour reaction added 100 µl of colour development solution to each well. Once again the wells were incubated at room temperature for 20 minutes. To stop the colour reaction, added 100 µl of the stop solution to each well. Read the absorbance at 450 nm. A semi log graph was constructed in MPM 3 software and the levels of IL-8 in the plasma samples were calculated.

Appendix 26
Estimation of Plasma Eosinophil Cationic Protein
Sandwich ELISA Kit Method (USCN, China)
(Reimert *et al.,* 1991)

Principle

The microtitre plate provided is pre-coated with an antibody specific to eosinophil cationic protein (ECP). Standards or samples are added. Next, avidin conjugated to horseradish peroxidase (HRP) is added and incubated. TMB substrate solution is added, only to those wells that contain ECP, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450nm. The concentration of ECP in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Reagents

1. Standard ECP: 39 – 2500 pg / ml
2. Detection reagent A
3. Detection reagent B
4. Wash solution
5. 3,3',5,5' – tetramethyl benzidine (TMB)substrate solution
6. Stop solution
7. Phosphate Buffered Saline (PBS) 0.02 M / L; pH 7.0-7.2.
8. Plasma: Plasma samples were diluted 200 fold with PBS

Procedure

The wells were laid for standard, blank and sample. In the ELISA Plate, 7 wells were used for standard of various range (2500, 1250, 625, 312, 156, 78, 39 pg / ml) 1 well for blank and the remaining wells for samples. 100 µl aliquots of seven different dilutions (S1 – S7) of standard, blank and samples were added into the appropriately laid wells followed by incubation for 2 hours at 37°C after covering the plate. Later, all the wells were decanted. Subsequently, added 100 µl of detection reagent A to all the wells followed by incubation for 1 hour at 37°C after covering with the plate sealer. Aspirated the solution from each well and washed thrice with 350 µl of wash solution. After the last wash, decanted the plate and blotted it against absorbent paper. Subsequently, 100 µl aliquots of detection reagent B was added to each well followed by incubation for 30 minutes at 37°C after covering with plate sealer. The wash process was repeated 5 times. Finally, added 90 µl of TMB substrate solution to each well. The plate was covered and incubated for 15 – 25 minutes at 37°C in dark. 50 µl of sulphuric acid stop solution was added to each well to stop the reaction. Read the absorbance at 450 nm immediately. Created a log-log standard graph using MPM3 software and extrapolated the results for plasma samples.

Appendix 27
Estimation of Plasma C-Reactive Protein
Sandwich ELISA Kit Method (Cayman Chemical, USA)
(Robey *et al.,* 1983)

Principle

This assay is based on a double antibody sandwich technique. Each well of the microtitre plate is coated with monoclonal antibody specific for human CRP (mouse anti-human CRP). Standards and samples are incubated on the antibody-coated plate followed by addition of Horse Radish Peroxidase (HRP) labeled CRP monoclonal antibody to detect the captured CRP. The two antibodies form a "sandwich" by binding to different locations on the CRP molecule. The concentration of the analyte is determined by measuring the enzymatic activity of HRP using the chromogenic substrate, tetramethyl benzidine. After a sufficient period of time, the reaction is stopped with acid, forming a product with a distinct yellow colour that can be measured at 450 nm. The intensity of this color, determined spectrophotometrically, is directly proportional to the amount of bound HRP labeled monoclonal antibody, which in turn is proportional to the concentration of the CRP.

Reagents

1. CRP assay buffer
2. Plasma: Plasma samples were diluted 1000 fold with assay buffer
3. Anti CRP HRP conjugate
4. CRP standard: 46.9 – 3000 pg / ml
5. 3,3',5,5' – tetramethyl benzidine (TMB) substrate solution
6. Stop solution

Procedure

100 µl aliquots of seven different dilutions (S1 – S7) of standard in the concentration range of 3000,1500,725,375,187.5,93.8,46.9 pg/ml, blank and samples were added into the appropriately laid wells followed by incubation for 1 hour at room temperature after covering the plate. Decanted the wells and rinsed four times with assay buffer followed by the addition of 100µl of anti CRP HRP conjugate to each well except the blank wells. Covered the plate with plastic film and incubated for 30 minutes at room temperature on a shaker. The wash procedure was repeated four times with assay buffer. After the last wash, the inverted plate was gently tapped on to the absorbent paper to remove the residual assay buffer. To initiate the colour reaction, added 100 µl of TMB substrate solution to each well. Covered the plate with plastic film and incubated for 15 minutes at room temperature in the dark on a shaker. 100 µl aliquot of stop solution was added to all the wells. Read the absorbance of the plate at 450 nm. Plotted the absorbance for standards versus CRP concentration using linear axis and fit the data with a quadratic equation. The CRP levels of plasma samples were calculated from this equation by using MPM3 software.

Appendix 28
Estimation of Plasma Total Nitrates
Greiss Method (Cayman Chemical, USA)
(Nims *et al.,* 1995)

Principle

In the first step of the assay, the nitrate in the sample is converted to nitrite utilizing nitrate reductase. The second step is the addition of the Greiss reagents which converts nitrite into a deep purple azo compound. The absorbance of the azo compound at 540 nm is directly proportional to the amount of total nitrites in the sample.

Reagents

1. Assay buffer
2. Nitrate reductase enzymes
3. Nitrate reductase co-factors mixture
4. Nitrate standard: 5 – 35 µM
5. Greiss reagent 1
6. Greiss reagent 2
7. Plasma: Plasma samples were diluted 2 fold with assay buffer

Procedure

200 µl of assay buffer was added to the blank wells. 80 µl of each standard ranging from 5 – 35 µM and of the diluted plasma samples were added to their corresponding laid wells followed by, the addition of 10 µl of the enzyme cofactor mixture and 10 µl of nitrate reductase was added to the standard and sample wells. The plate was covered with a plate sealer and incubated at room temperature for 3 hours. 50 µl of Greiss reagent 1 was added to the standard and sample wells. 50 µl of Greiss reagent 2 was added to the standard and sample wells. The colour was allowed to develop for 10 minutes at room temperature. Read the absorbance at 540 nm using a plate reader. The sample nitrate values were extrapolated from the linear plot of absorbance at 540 nm as a function of standard nitrate concentration.

Appendix 29
Estimation of Plasma Myeloperoxidase Activity
Sandwich ELISA Kit Method (Enzo Life Sciences, Switzerland)
(Falk and Jennette, 1988)

Principle

A monoclonal antibody to myeloperoxidase (MPO) immobilized on a microtitre plate binds the MPO in the standards or sample. After a short incubation, the excess sample or standard is washed out and a rabbit polyclonal antibody to MPO is added. This antibody binds to the MPO captured on the plate. After a short incubation, the excess antibody is washed out and goat anti-rabbit IgG conjugated to horseradish peroxidase is added, which binds to the polyclonal MPO antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the colour generated is read at 450 nm. The measured optical density is directly proportional to the activity of MPO in either standards or samples.

Reagents

1. Human MPO Antibody: Rabbit polyclonal antibody to MPO.
2. Assay Buffer: Citrate buffered saline containing detergents.
3. Human MPO Conjugate: Goat anti-rabbit IgG conjugated to Horseradish peroxidase.
4. Wash buffer: Tris buffered saline containing detergents.
5. Human MPO Standard: 125 ng / ml : Diluted to 0.195 – 12.5 ng / ml
6. TMB Substrate: 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide
7. Stop Solution: 1N HCl
8. Plasma: Lithium heparin plasma was diluted 24 fold.

Procedure

Into the blank well pipetted 100 µl of standard diluents, and into standard wells, (S1 - S7) added 100 µl aliquots of standards in various dilution ranging from (12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.195 ng/ ml) and into other wells added 100 µl aliquots of samples. Sealed the plate and incubated at room temperature on a plate shaker for 1 hour at approximately 500 rpm. Emptied the contents of the wells and washed with 400 µl of wash solution in every well. The wash procedure was repeated 3 more times. After the final wash, aspirated the wells and firmly tapped the plate on a lint free paper towel to remove any remaining wash buffer. In the next step, added 100 µl of rabbit polyclonal antibody into each well except the blank. Sealed the plate and incubated at room temperature on a plate shaker for 1 hour at approximately 500 rpm. The wash procedure was repeated 4 times as before. After the final wash, aspirated the wells and firmly tapped the plate on a lint free paper towel to remove any remaining wash buffer. Added 100 µl of goat anti-rabbit IgG conjugated to horseradish peroxidase to each well, except the blank. Sealed the plate and incubated at room temperature on a plate shaker for 30 minutes at approximately 500 rpm. The wash procedure was repeated 4 times as before. After the final wash, aspirated the wells and firmly tapped the plate on a lint free paper towel to remove any remaining wash buffer. 100 µl aliquots of substrate solution was added into each well. Sealed the plate and incubated for 30 minutes at room temperature on a plate shaker at approximately 500 rpm. To stop the colour reaction added 100 µl of stop solution to each well. Read the optical density at 450 nm. A standard curve was drawn using a 4 parameter logistic fit graph by using MPM3 software.

Appendix 30
Estimation of Plasma Intercellular Adhesion Molecule-1
Sandwich ELISA Kit Method (USCN, China)
(Rothlein *et al.,* 1988)

Principle

The microtitre plate is pre-coated with an antibody specific to Intercellular Adhesion Molecule (ICAM1). Standards or samples are added to the appropriate wells with a biotin-conjugated antibody preparation specific for ICAM1. Avidin conjugated to Horseradish Peroxidase (HRP) is added and incubated. Then, TMB substrate solution is added. Only those wells that contain ICAM1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450nm. The concentration of ICAM1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Reagents

1. Standard ICAM1: 78 – 10,000 pg / ml
2. Detection reagent A: Biotin conjugated antibody
3. Detection reagent B: Avidin conjugated horseradish peroxidase
4. Wash buffer
5. Substrate solution: 3,3',5,5' – tetramethyl benzidine (TMB)
6. Sulphuric acid
7. Plasma: Samples were diluted 250 fold in PBS before addition to wells.

Procedure

The wells were laid for standard, blank and sample. In the ELISA Plate, 7 wells were used for standard of various range (5000, 2500, 1250, 625, 312, 156, 78 pg /ml) 1 well for blank and the remaining wells for samples. Added 100 µl aliquots of seven dilutions of (S1 – S7) standard, blank and samples into the appropriate wells followed by incubation, for 2 hours at 37°C after covering the plate. Later, all the wells were decanted. Subsequently, added 100 µl of detection reagent A to all the wells followed by incubation for 1 hour at 37°C after covering with the plate sealer. Aspirated the solution from each well and washed thrice with 350 µl of wash solution. After the last wash, decanted the plate and blotted it against absorbent paper. Subsequently, 100 µl aliquots of detection reagent B was added to each well followed by Incubation for 30 minutes at 37°C after covering with plate sealer. The wash process was repeated 5 times. Finally, added 90 µl of TMB substrate solution to each well. The plate was covered and incubated for 15 – 25 minutes at 37°C in dark. 50 µl of sulphuric acid stop solution was added to each well to stop the reaction. Read the absorbance at 450 nm immediately. Created a log-log standard graph using MPM3 software and extrapolated the results for plasma samples.

Appendix 31
Estimation of Plasma Glutathione peroxidase Activity
Colorimetric Kit Method (Cayman Chemical, USA)
(Paglia and Valentine, 1967)

Principle

Glutathione peroxidase (GPx) activity is measured indirectly by a coupled reaction with glutathione reductase (GR). Oxidised glutathione (GSSG), produced upon reduction of hydroperoxide by GPx is recycled to its reduced state by GR and NADPH:

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPx}} \text{R-O-H} + \text{GSSG} + H_2O$$

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

Reagents

1. Assay buffer: 50 mM Tris- HCl, 5 mM EDTA, pH 7.6
2. Sample buffer: 50 mM Tris-HCl, 5 mM EDTA, 1 mg / ml BSA, pH 7.6
3. GPx standard
4. GPx co substrate mixture: NADPH, glutathione and glutathione reductase
5. Cumene hydroperoxide
6. Plasma

Procedure

120 µl of assay buffer and 50 µl of co-substrate mixture were added to the blank wells. 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of diluted GPx were added to the standard wells. To the sample wells, 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of sample were added. The reaction was initiated on addition of 20 µl of cumene hydroperoxide to all the wells. Read the absorbance once every minute at 340 nm for 5 time points.

Calculations

The absorbance values were plotted as a function of time to obtain the slope of the linear portion of the curve. Selected two points on the linear position of the curve and determined the change in absorbance during that time using the following equation:

ΔA_{340} / minutes = [A_{340} (Time 2) - A_{340} (Time 1)] / Time 2 (min) – Time 1 (min).

Extinction coefficient of NADPH at 340 nm for 0.6 cm path length is 0.00373 μM^{-1}

The rate of A_{340} / minutes is calculated by the following formula:

GPx activity (nmol / minutes / ml) = [ΔA_{340} / minutes / 0.00373 μM^{-1}] x [0.19 ml / 0.02 ml]

Appendix 32
Estimation of Plasma Thioredoxin reductase Activity
Colorimetric assay kit (Cayman Chemical, USA)
(Luthman and Holmgren, 1982)

Principle

Thioredoxin reductase (TrxR) activity is detected based on the reduction of DTNB (5, 5'-dithio-bis (2-nitrobenzoic acid) with NADPH to 5-thio-2-nitrobenzoic acid (TNB) which produces an yellow product that is measured at 415 nm. Measurement of TrxR activity by DTNB reduction in the presence and absence of aurothiomalate (ATM), a specific TrxR inhibitor allows for correction of non-thioredoxin reductase independent DTNB reduction. The difference between the two results in the DTNB reduction is due to TrxR activity.

$$TrxR + DTNB + NADPH + H^+ \longrightarrow 2TNB + NADP^+$$

Reagents

1. Assay buffer: 50 mM potassium phosphate, 50 mM KCl, 1 mM EDTA, 0.2 mg / ml BSA, pH 7.0
2. Standard TrxR
3. TrxR inhibitor: 20 µM aurothiomalate (ATM)
4. 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB): 4 mg in 2 ml Dimethyl Sulfoxide (DMSO)
5. NADPH
6. DMSO

Procedure

All samples were assayed for the reduction of DTNB in the presence and absence of inhibitor. The wells were appropriately laid as blank, blank+inhibitor wells, standard wells and sample wells. To the blank wells, added 160 µl of assay buffer. To the blank + inhibitor wells, added 140 µl of assay buffer and 20 µl of aurothiomalate. To the standard wells, added 140 µl of assay buffer and 20 µl of standard TrxR. To the sample wells, added 140 µl of assay buffer and 20 µl of sample. To the sample + inhibitor wells, added 120 µl of assay buffer, 20 µl of aurothiomalate and 20 µl of sample. The reactions were initiated by the addition of 20 µl of NADPH and 20 µl of DTNB to all the wells. Read the absorbance once every minute at 415 nm to obtain five time points.

Calculation

The absorbance values were plotted as a function of time to obtain the slope of the linear portion of the curve. Selected two points on the linear position of the curve and determined the change in absorbance during that time using the following equation:

ΔA_{415} / minutes = [A_{415} (Time 2) - A_{415} (Time 1)] / Time 2 (min) – Time 1 (min).

The rate of A_{415} / minutes is calculated by the following formula:

Corrected ΔA_{415} / minutes (sample) = ΔA_{415} / minutes (sample) – [ΔA_{415} / minutes (sample + ATM) - ΔA_{415} / minutes (Blank + ATM)]

TrxR activity (µM / minutes / ml) =

[Corrected ΔA_{415} / minutes (sample) / 6.35 mM^{-1}] x [0.2 ml / 0.02 ml]

Appendix 33
Estimation of Serum Cadmium and Selenium
(Inductively Coupled Plasma Optical Emission Spectroscopy)

Principle

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) is principally an emission spectroscopy. When the sample is introduced into the argon plasma induced by radiofrequency, it gets nebulized and then enters into the plasma which contains both positive and negative charges. The sample on collisional excitation with high energy plasma gets desolvated, later on is vaporized, atomized and ionised and the electrons get excited to go to higher energy levels. On return to the ground state, it will emit excess energy in the form of electromagnetic radiation, which is sensed by the detector. In ICP-OES, the intensity of emitted electromagnetic radiation at specific wave length measured is directly proportional to the concentrations of the metal element (Boss and Fredeen, 1997).

Procedure

The ICP experiments were performed using Perkin Elmer 5300DV Optical Emission Spectrometer equipped with a Gem Tip Cross - Flow nebulizer, Scott - type spray chamber, peristaltic pump, argon – supported inductively coupled plasma and charge coupled (UV-Visible) detector maintained at - 40^0 C, with compressed gas as shear gas. High pure nitrogen gas was used to create vacuum in the detector. The instrument was calibrated with 2% nitric acid solution. Then standards were run and then reagent blank was run to complete the calibration of the instrument. The diluted serum sample in the ratio of 1: 3 was introduced into the instrument through the peristaltic pump, as a stream of clear aqueous solution with the following instrument settings: plasma flow rate: 15 l / minute, auxillary flow rate: 0.2 l / minute, nebulizer flow rate: 0.80 l / minute, pump rate: 1.5 ml / minute, rf power: 1300 watts, sample uptake rate: 1.5 ml / minute, sample delay time: 60 seconds. All the samples were analyzed at 228.8 nm for Cadmium and 196 nm for Selenium with a read delay of 80 second and an integration time from 2 - 5 second. The software used in the instrument for the analysis was “WINLAB32”.

Appendix 34
Assessment of Genotoxicity
Comet Assay in Whole Blood
(Dhawan *et al.,* 2009)

Principle

Comet assay is a sensitive method to detect DNA damage. Molten agarose embedded cells are lysed with high detergent and salt and the liberated DNA is electrophoresed under alkaline condition. Cells with increased DNA damage shows altered migration of the DNA towards the anode forming a tail. The undamaged DNA remains linked to the nuclear matrix forming head. This pattern resembles a comet. The intensity of the comet tail relative to the head reflects the DNA damage.

Reagents

1. Phosphate Buffered Saline (PBS)
2. Normal melting point agarose (NMPA): 1 %
3. Low melting point agarose (LMPA): 0.1 %

4. Lysing Solution: 2.5 M NaCl; 100 mM Ethylene diamine tetraacetate (EDTA); 10 mM Trizma base; 8 g NaOH; pH 10.0 Final lysing solution: added fresh 1% Triton X-100 and 10% DMSO,
5. Electrophoresis Buffer: 300 mM NaOH / 1 mM EDTA, pH 13.0
6. Neutralization Buffer: 0.4 M Tris, pH 7.5
7. Staining Solution: Ethidium bromide - 20 µg / mL

Base Slide Preparation

The oil and grease free slides were dipped up to one-third in to the hot NMPA, and were gently removed. After wiping on the underside, the slides were dried on a flat tray. The slides were stored at room temperature until the usage for the next day.

Cell Treatment

To the coated slide, added 80 µl of diluted blood (1: 1 dilution with PBS) and equal volume of molten 1% LMPA. The coverslip was placed and the slide was put on a slide tray resting on ice packs until the agarose layer hardens (~5 to 10 minutes). The coverslip was gently slided off and a third agarose layer was added (80 µL LMPA).The coverslip was then replaced and returned to the slide tray until the agarose layer hardens (~5 to 10 minutes). The coverslips were removed and slowly lowered the slide into cold, freshly made lysing solution in jars. They were protected from light and refrigerated for a minimum of 2 hours.

Electrophoresis

After at least 2hour at ~4ºC, the slides were removed from the lysing solution and the slides were placed and arranged side by side in the gel box, as close together as possible. The buffer reservoir was filled slowly with freshly made electrophoresis buffer pH>13 till the slides got immersed in the buffer. The slides were laid in the buffer for 20 minutes to allow for unwinding and the expression of alkali-labile damage of DNA. The power supply was turned on to 24 volts (~0.74 V/cm) and adjusted the current to 300 milliamperes by raising or lowering the buffer level. The slides were electrophoresed for 30 minutes. The power was then turned off. The slides were then gently lifted from the buffer and placed on a drain tray. The slides were then treated with neutralization buffer drop by drop for 5 minutes. The slides were drained and repeated the procedure for twice. The slides were then kept for 20 min in cold 100% ethanol for dehydration. The slides were then air dried. While viewing, they were rehydrated with chilled distilled water for 30 minutes and were stained with 80µL of 1X Ethidium bromide, left for 5 min and then dipped in chilled distilled water to remove excess stain. The coverslip was then placed over it and the slides were blotted on the back and edges before viewing and were then scored. After scoring of the comet cells, removed the coverslip, rinsed in 100% alcohol to remove stain, let dry, and stored for archival purposes.

Evaluation of DNA Damage

For visualization of DNA damage, observations were made on EtBr-stained DNA using a 40x objective on a fluorescent microscope (Nikon, Japan). The slides were viewed by fluorescent microscopy at 40X magnifications. The image analysis software (Tritek Corp v1.5, USA) was used for the quantification of SCGE data linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. 50 cells were selected and scored for the analysis per sample. Finally, the program calculates % DNA in the comet tail.

Appendix 35
Metabolomic Study of Urine by Nuclear Magnetic Resonance Spectroscopy
(**Ramadan *et al.,* 2006**)

Principle

NMR spectroscopy studies of molecules are done by recording the interaction of a radiofrequency electromagnetic radiation with the nuclei (e.g., ^{1}H, ^{13}C, ^{15}N, etc.) placed in a strong magnetic field. A single nucleus in a molecule can be “observed” by monitoring the corresponding line (a “resonance”) in an NMR spectrum and the various parameters of that line

(frequency, splitting, line width and amplitude) can be used to determine the molecular structure, conformation and dynamics of the molecule. In principle, assignment (i.e., identification) of NMR resonances for common metabolites could be possible by comparing the observed chemical shifts (i.e., the position of the line in a spectrum) with published reference data.

Reagents

1. D_2O
2. Sodium azide

Procedure

Urine ^{1}H NMR Procedure

Morning spot urine (20 ml) during work hours were collected from the participants and stored in urine containers with sodium azide as preservative. They were frozen immediately and stored at - 80ºC. The samples were thawed once in safety fume hood for the analysis. To 630 µl of urine aliquot was added to the reference phosphate buffer 0.2 M Na_2 HPO_4 / 0.2 M NaH_2PO_4 pH 7.4, 80% H_20, 20% D_2O in the ratio of 3:1 and centrifuged in 1.5 ml cryovial. The samples were subsequently centrifuged and filled in NMR tubes. All one dimensional ^{1}H NMR spectra were acquired on a Burker AV III 500 MHz at 298K. 1D spectra were acquired with water suppression. For each spectrum, 64 transients were collected using 32000 data points and a 6000 Hz spectral width. An exponential weighting function corresponding to 0.3 Hz line broadening was applied to the free induction decay before applying Fourier transformation. All spectra were phased and baseline corrected using Bruker's XWINNMR software. Each NMR spectral region was reduced to smaller number of variables by exclusion to eliminate variation in water suppression efficiency peaks <0.4, >9.0, 4.5- 5.1 (water peak), and 7.378 - 7.480, 8.467 - 8.679 (imidazole peaks). NMR spectra were collected for total of 40 participants (unexposed group n= 20, exposed group n= 20).