Chemicals

7,12-dimethylbenz(a)anthracene (DMBA), Sinapic acid, Reduced glutathione and Reduced nicotinamide adenine dinucleotide, 1,1',3,3'-tetramethoxy propane, were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Heparin, Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), 2,4-dinitro phenyl hydrazine (DNPH), 5,5'-dithiobis 2-nitro benzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), Nitroblue tetrazolium (NBT), Phenazine methosulphate (PMS) were purchased from Himedia laboratories, Mumbai, India. p53, Bcl-2, Bax and Caspase-3 primary antibodies were purchased from Dako, Carprinteria, CA, USA. Power Block^{TM} reagent and secondary antibody conjugated with horseradish peroxidase were purchased from BioGenex, San Ramon, CA, USA.

In Vitro Studies

Free radical scavenging activity

The DPPH Free radical scavenging activity of Sinapic acidwas determined by the method of Yen and Hsieh, (1995). Antiradical activity assay is based on the reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Due to the presence of an odd electron it gives a strong absorption maximum at 517 nm.

Reagent

1. 2,2-Diphenyl-1-picryl hydrazyl $(DPPH)^{\bullet}$ – 90.25 mM in methanol in a dark room.

Procedure

To a methanolic solution of DPPH (90.25 mM), an equal volume of Sinapic acid (10-50 µM) was added and made up to 1.0 mL with methanolic DPPH. An equal amount of methanol was added to the control. After 20 min, the absorbance was recorded at 517 nm in a systronics UV-Visible spectrophotometer. Ascorbic acid was used as standard for comparison. The inhibition of free radicals by DPPH in percentage terms (%) was calculated by using the following equation.

$$\% \text{ Scavenging} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 absorbance of blank, A_1 absorbance of standard Sinapic acid

Total antioxidant activity

Total antioxidant potential of Sinapic acid was determined by the ABTS assay as described by Re *et al.,* (1999).

Reagents

1. 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)($ABTS^{+}$) -0.002 M
2. Phosphate buffer – 100 mM, pH 7.4

Procedure

The reaction mixture contained ABTS (0.002 M), Sinapic acid (10-50 µM) and buffer in a total volume of 3.5 mL. The absorbance was measured at 734 nm and compared with ascorbic acid at various concentrations (10-50 µM). The percentage of inhibition was calculated.

$$\% \text{ Scavenging} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 absorbance of blank, A_1 absorbance of standard Sinapic acid

Superoxide anion scavenging activity

Superoxide anion scavenging activity of Sinapic acid was determined by the method of Nishmiki *et al.,* (1972) with modifications. The assay was based on the superoxide anion generation from dissolved oxygen by PMS-NADH coupling reaction and reduces NBT. Oxidation of NADH by PMS to liberate PMS_{red}, which in turn converts oxidized nitroblue tetrazolium (NBT_{oxi}) to the reduced form NBT_{red}. This forms a violet colour complex indicating the generation of superoxide anion, which was measured spectrophotometrically at 560 nm. A decrease in the formation of color after addition of the antioxidant was a measure of its superoxide radical scavenging activity.

Reagents

1. Methanol
2. Phosphate buffer – 100 mM, pH 7.4

3. Nitroblue tetrazolium (NBT) – 100 µmol of NBT in phosphate buffer
4. Reduced nicotinamide adenine dinucleotide (NADH) -14.68 µmol of NADH in phosphate buffer
5. Phenazine methosulphate (PMS)- 60 µmol/100mmol in phosphate buffer

Procedure

1 mL of NBT, 1 mL of NADH solution and varying volumes of Sinapic acid (10-50 µM) were mixed well. The reaction was started by the addition of 100 µM of PMS. The reaction mixture was incubated at 30ºC for 15 min. The absorbance was measured at 560 nm in a spectrophotometer. Incubation without Sinapic acid was used as blank. Ascorbic acid was used as standard for comparison. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging. The % of inhibition was calculated as shown below:

$$\% \text{ Scavenging} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 absorbance of blank, A_1 absorbance of standard, Sinapic acid

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was determined by the method of Aruoma, (1989). In this assay, hydroxyl radicals are produced by the reduction of H_2O_2 by the transition metal (iron) in the presence of ascorbic acid. The generation of hydroxyl radical is detected by its ability to degrade deoxyribose to form products, which on heating with TBA forms a pink colour chromogen. Addition of Sinapic acid competes with deoxyribose for hydroxyl radicals and diminishes the colour formation.

Reagents

1. Potassium phosphate buffer - 100 mM, pH 6.7
2. Ferric chloride - 500 mM in buffer
3. Ascorbic acid - 1 mM in buffer

4. Ethylene diamine tetra acetate (EDTA) - 1 mM in buffer
5. Hydrogen peroxide (H_2O_2) - 10 mM
6. 2-Deoxyribose - 15 mM in buffer
7. Thiobarbituric acid (TBA) - 1% in 0.05N Sodium hydroxide
8. Trichloro acetic acid (TCA) - 28% in water

Procedure

The following reagents were added in the order stated below. The incubation mixture in a total volume of 1 mL contained 0.2 mL of potassium dihydrogen phosphate buffer, varying concentrations of Sinapic acid (10-50 µM), 0.2 mL of ferric chloride, 0.1 mL of ascorbic acid, 0.1 mL of EDTA, 0.1 mL of H_2O_2 and 0.2 mL of 2-deoxy ribose. The contents were mixed thoroughly and incubated at room temperature for 60 min. Then 1 mL of TBA and 1 mL of TCA were added. All the tubes were kept in a boiling water bath for 30 min. The absorbance was read in a spectrophotometer at 532 nm with reagent blank containing distilled water in a place of Sinapic acid. The efficiency of Sinapic acid was compared with various concentrations (10-50 µM) of standard ascorbic acid (Vitamin C). Decreased absorbance of the reaction mixture indicated increased hydroxyl radical scavenging activity. The percentage of scavenging activity was determined as shown in below:

$$\% \text{ Scavenging} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 absorbance of blank, A_1 absorbance of standard Sinapic acid

Nitric oxide radical scavenging activity

The nitric oxide radical inhibition activity was measured by the method of Garrat, (1964). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide that interacts with oxygen to produce nitrite ions, which can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions.

Reagents

1. Sodium nitroprusside 10mM
2. Griess reagent

Procedure

Sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations of Sinapic acid(10-50 µM), dissolved in methanol and incubated at room temp for 150 min. After the incubation period, 0.5 mL of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-cl-(napthyl.)-Ethylene-diamine dihydrochloride) was added. The reaction mixture without Sinapic acid served as the control. The absorbance of the chromofore formed was read at 546 nm. The percentage of scavenging activity was determined as shown in below:

$$\% \text{ Scavenging} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 absorbance of blank, A_1 absorbance of standard Sinapic acid

Reducing power

The reducing power was determined according to the method of Yen and Chen, (1995) with slight modification. Substances which have reduction potential react with potassium ferric cynaide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. Increase in the reduction of ferric to ferrous ion increases the absorbance indicating the reducing ability of sinapic acid.

Reagents

1. Phosphate buffer - 0.2 M, pH 6.6
2. Potassium ferricyanide - 1% in water
3. Trichloroacetic acid (TCA) - 10% in water Ferric chloride - 0.1 w/v in water

Procedure

Different concentrations of sinapic acid (10-50 µM) were prepared in methanol mixed with 2.5 mL of phosphate buffer and 2.5 mL of potassium ferricyanide. The mixture was incubated at 50°C for 20 min after which 1.5 mL of TCA was added and centrifuged at 3000xg for 10 min. From all the tubes, 0.5 mL of supernatant was mixed with 1mL of distilled water and 0.5 mL of ferric chloride. The absorbance was measured at 700 nm in a spectrophotometer. Ascorbic acid was used as standard for comparison. Increased absorbance of the reaction mixture indicated increasing reducing power. Incubation with water in place of additives was used as the blank. The reducing power activity was determined as shown in below:

$$\% \text{ Scavenging} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 absorbance of blank, A_1 absorbance of standard, sinapic acid

***IN VITRO* CYTOTOXICITY STUDIES**

Tumor cell line

The present work was carried out in human oral cancer cell line (Larynx) of Human epidermoid carcinoma cell line (HEp-2). This cell line was obtained from National Centre for Cell Science (NCCS), Pune, India.

Culture medium

Medium

Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate was used for growing the cells and was obtained from Himedia Laboratories, Mumbai, India.

Serum

Fetal bovine serum was used for the growth of cells was obtained from Himedia Laboratories, Mumbai, India.

Maintenance of cells

The HEp-2 cells were grown as monolayer in Minimum essential medium (MEM) supplemented with 10% FBS, 1% glutamine, and 100 U/mL penicillin-streptomycin at 37°C in 5% CO_2 atmosphere. Stocks were maintained in 25cm^2 tissue culture flask.

Characteristic features of HEp-2 cell line

Designation	: HEp-2
Organism	: Homo sapiens (human)
Tissue	: Larynx
Morphology	: Epithelial
Cell type	: Epidermoid carcinoma
Growth Properties	: Monolayer
Description	: The cells are positive for keratin by immunoperoxidase staining.
Culture medium	Minimum essential medium (Eagle) supplemented with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids 1.0 mM sodium pyruvate and 10% fetal bovine serum
Subculturing	Remove the medium, add fresh 0.025% trypsin or 0.02 % EDTA solution rinse and remove trypsin. Allow flask to sit at room temperature (or at 37°C) until cells detach. Add fresh medium, aspirate and dispense into new flasks.
Split ratio	: A ratio of 1:4 to 1:10 is recommended
Fluid renewal	: 2 to 3 times weekly
Karyotype	: Hypertriploid, with abnormalities including dicentrics, breaks, pulverizations and minutes
HeLa marker	: Yes

Cell counting

Materials and equipments

0.04 % trypan blue in PBS, haemocytometer and inverted microscope.

Method

The cell numbers are counted, cells were seeded at 5 x 10^4 cells per well in 24-well plates. Cells were harvested by trypsinization. The cell suspension was mixed gently and aliquots was added to the trypan blue solution-I (100 µL cell suspension: 100 µL dye) and was then counted in haemocytometer.

Calculations

Total no. of viable cells	=	$A \times B \times C \times 10^4$
Total no. of dead cells	=	$A \times B \times D \times 10^4$
Total cell count	=	Viable cell count + dead cell count
% Viability	=	Viable cell count×100/total cell count

Where, A = volume of cells

B = dilution factor in trypan blue

C = mean number of unstained cells

D = mean number of dead cells or stained cells

10^4= Conversion factor for 0.1 mm^3 to1 mL

Drug preparation

Sinapic acid was prepared as fresh solution with DMSO and stored at -20ºC. The stock solution was diluted with sterile MEM medium to arrive at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM of Sinapic acid was used for further studies.

Stock solution

5 mL Sinapic acid in 5mL DMSO

Working solution

100 µL of stock solution contain 100 µg

of Sinapic acid with 900µL of medium = 1 mL working solution (1 µg/1µL).

MTT Assay

The growth activity of cell populations were untreated and treated with Sinapic acid in different concentration such as 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM was determined by the MTT assay based on the detection of mitochondrial dehydrogenase activity in living cells (Ferrari, 1990).

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) Assay, in which the yellow tetrazolium salt is metabolized by NAD-dependent dehydrogenase (in active mitochondria) to form a dark blue formazan product. Yellow color MTT is converted to the blue formazan product only by metabolically active mitochondria, and the absorbance is directly proportional to the number of viable cells and inversely proportional to the degree of cytotoxicity.

Reagents

1. 5 mg/mL of 1x PBS MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide)
2. Dimethyl sulfoxide (DMSO)

Preparation

Stock solution

50 mg of MTT was dissolved in 10 mL of PBS (5 mg / mL). The mixture was vortexes for 20 minutes; it was filtered through 0.45 micron filter. The bottles were wrapped with aluminium foil to block light, as MTT is light sensitive. The preparation was stored at 4ºC.

Procedure

HEp-2 oral cancer cells were seeded in 96 well microtiter plate (5×10^3 cells/well) and incubated for 24 h and 48 h. The cells were incubated with Sinapic acid at different concentrations ranging from 10-100 µM of Sinapic acid for 24h and 48 h. The untreated cells serve as control. MTT solution (5 mg / mL) was added to each well in the 96 well plate and formazan blue color was allowed to develop for additional 4 h incubation. An equal volume of DMSO was added to stop the reaction and to solubilize the blue crystals. Samples were transferred into culture plates and the absorbance was measured colorimetrically at 540 nm.

Measurement of intracellular ROS in cells by fluorescence microscopy

ROS was measured by using a non-fluorescent probe, 2, 7,-diacetyl dichlorofluorescein diacetate (DCFH-DA) that can penetrate into the intracellular matrix of cells where it is oxidized by ROS to fluorescent dichlorofluorescein (DCF). The non-fluorescent DCFH-DA is oxidized by intracellular ROS and forms the highly fluorescent DCF observed microscopically (Jesudason *et al.,* 2008)

Reagents

1. Phosphate buffered saline (PBS)
2. 2-7-diacetyl dichlorofluorescein (DCFH-DH)

Procedure

The percentage of ROS was estimated in the control and Sinapic acid treated cancer cells (IC_{50} for 24 h and 48 h). Briefly, an aliquot of the above mentioned isolated cells 8 x 10^6 cells / mL were made up to a final volume of 2mL in normal phosphate buffered saline (pH 7.4). An 1 mL aliquot of cells were taken, to which 100 µL DCFH-DA (10 µM) was added and incubated at 37°C for 30 min. we have observed the cells microscopically using blue filter.

Changes of mitochondrial transmembrane potential

Alteration in mitochondrial membrane potential (Depolarization) is an indication of early stages of apoptosis. Rhodamine 123 (Rh 123) is a lipophilic cationic dye, highly specific for mitochondria. Polarized mitochondria were marked by orange-red fluorescence and depolarized mitochondria were marked by green fluorescence (Philip Jesudason *et al.,* 2008).

Materials

1. Stock solution - Rhodamine 123 (Rh 123) stain- 1 mg of the dye in 1mL of PBS
2. Working solution - Working – 10 µL from stock and made up to 1 mL with PBS

Procedure

The cells 1x10^6 cells / mL were cultured in 6-well plate and treated with IC_{50} concentration of Sinapic acid for 24 h and 48 h and the untreated cells serves as control. The cells were then stained with Rhodamine 123 (Rh 123) dye (10 µg/mL) and the cells were kept incubation for 30 minutes in CO_2 incubator. The cells were

washed by the addition of warm PBS and the mitochondrial depolarization patterns of the cells were observed in the fluorescence microscope using blue filter.

Assessment of apoptotic and morphological changes by Acridine Orange/ Ethidium Bromide (DUAL STAINING METHOD)

In the dual staining of cells the apoptotic nuclei exhibits typical changes such as nuclear condensation and segmentation (Lakshmi *et al.*, 2008). Sinapic acid treated and untreated cells (5 x 10^3 /well) were seeded into 6-well plate and incubated in CO_2 incubator for 24 h and 48 h and then the apoptotic morphological changes were observed using a fluorescence microscope under blue filter.

Reagents

1. Phosphate buffered saline (PBS)
2. Acridine orange (AO)
3. Ethidium Bromide (EBr)

Procedure

The HEp-2 cells were grown in 6-well plates (5×10^3) for 24 h and 48 h and then treated with IC_{50} concentration of Sinapic acid were incubated in CO_2 incubator for 24 h and 48 h. The medium was discarded and the cells were washed in PBS. The cells were trypsinized and stained with 1:1 ratio of AO/EBr. Stained cells were immediately washed again with PBS and viewed under a fluorescence microscope with a magnification of 40x.

Cell cycle analysis

Flow cytometer- based DNA content analysis is an important tool to detect cell death/ cell cycle arrest. The DNA content distribution of normal cell characterized by the two peaks – G_1/G_0 and G_2/M phase. G_1/G_0 phase possesses normal functioning and resting state of cell cycle with most diploid DNA content, while the DNA content in G_2/M phase are more than diploid. Cells in sub G_1 phase have least DNA content in the cell cycle distribution is called hypodiploid. The hypodiploid DNA content represents the DNA fragmentation (Chakraborty *et al.*, 2012).

Reagents

1. Phosphate buffered saline (PBS)
2. Trypsin

3. 70% Ice cold ethanol
4. Propidium iodide
5. RNase
6. EDTA
7. Tween-20

Procedure

Cells are seeded T25 flask at a density of 1×10^6 cells/flask. After 24 h and 48 h, IC_{50} concentration of Sinapic acid was added to each flask incubated for 24 h and 48 h. The cells were trypsinized, harvested and fixed in 70% ice cold ethanol in cell culture tubes and stored at -20°C until use (within 7 days). The cells were centrifuged, the cell pellets were resuspended PI (40 µg/mL in PBS) solution containing RNase (100 µg/mL). The stained cells were analyzed using fluorescence activated cell sorter (FACScan, Becton- Dickinson) with 488 nm argon ion laser using MAC Cell-Quest™ Software. The cell cycle distribution was analyzed using PI signals were collected using the 585/42 band pass filter. The data acquired were analyzed using quest software.

IN VIVO STUDIES

Animals

Eight to ten weeks old male golden Syrian hamsters (*Mesocricetus auratus)* weighing 80-120 g were purchased from National Institute of Nutrition, Hyderabad, India. The hamster were housed in polypropylene cages and allowed to free access to food and water (*ad libitum*) throughout the experimental period. The hamster was maintained under controlled conditions of temperature 23 ± 2°C, and a light/dark (12h/12 h) cycle (Regn. No 1416/a/11/CPCSEA) (Muthayammal College of Arts and Science, Rasipuram, India). The animals were maintained under the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with the Indian National Law on animal care and use.

Tumor inductions

Oral squamous cell carcinoma was developed in the buccal pouch of male Syrian golden hamsters by painting with 0.5% DMBA in liquid paraffin three

times a week for 16weeks. The total numbers of tumor in the hamster's buccal pouch were counted. The diameter of the each tumor was measured by using vernier Caliper. The tumor volume was calculated using the formula $V = \frac{4}{3}\pi\left(\frac{D1}{2}\right)\left(\frac{D2}{2}\right)\left(\frac{D3}{2}\right)$ where D_1, D_2, and D_3 are the three diameters (in cm) of the tumor.

Experimental designs

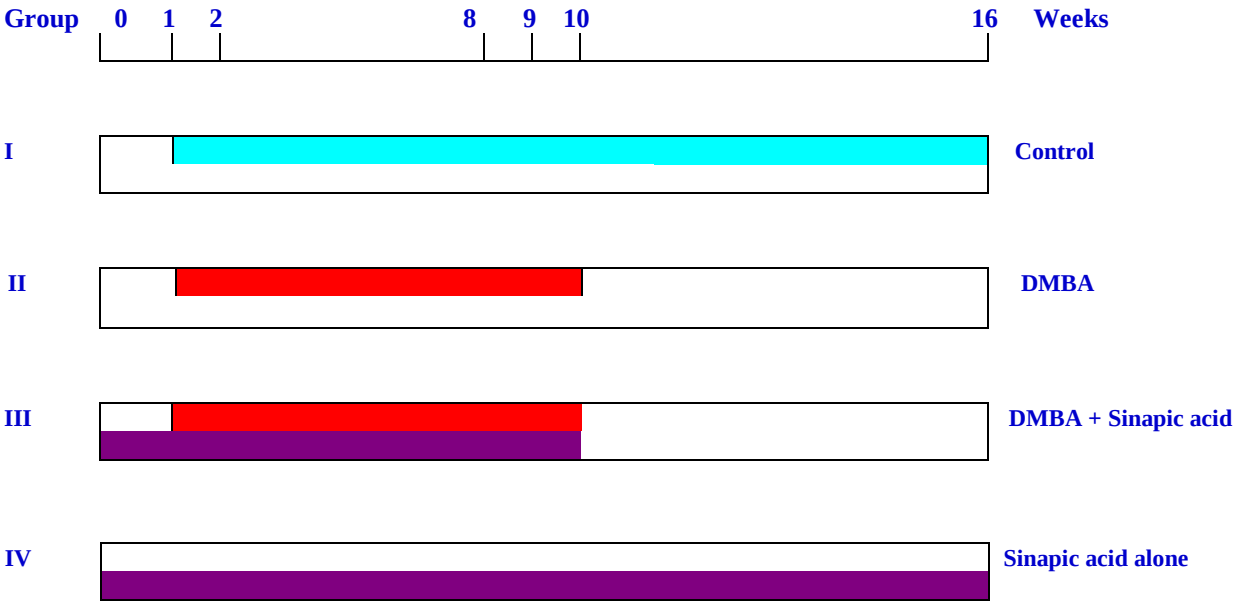
Dose dependent study

The experiments were designed and conducted in accordance with the institutional ethical guidelines. Male hamsters, 8-10 weeks old, weighing 95-125g was randomized in to control and experimental groups. A total number of 48 hamsters were divided into 6 groups of 8 animals each. Group 1 animal were served as untreated control. Animals in groups 2 through 5 were induced oral carcinogenesis by painting with DMBA in liquid paraffin three times a week for 16 weeks. Group 2 received no other treatment. Group 3-5 hamsters were orally administered with sinapic acid at a dose of 25, 50 and 100 mg/kg b.wt respectively, starting 1 week before the exposure to the carcinogen and continued until 1 week after the final exposure to the carcinogen. Group 6 animals were orally administered with sinapic acid alone at a concentration of 50 mg/kg b.wt throughout the experimental period. The experiment was terminated at the end of 16^{th} week to find out the effective dose of sinapic acid.

Chemoprevention study

A total number of 32 hamsters were divided into 4 groups of 8 animals each. Group 1 animals were served as untreated control. Animals in groups 2 and 3 were induced oral carcinogenesis by painting with DMBA in liquid paraffin three times a week for 16 weeks. Group 2 received no other treatment. Group 3 were orally administered with sinapic acid (50mg/animal/day), starting 1 week before the exposure to the carcinogen and continued until 1 week after the final exposure to the carcinogen (pre-initiation phase). Groups 4 animals were orally administered with sinapic acid (50mg/animal/day) respectively. The experiment was terminated at the end of 16^{th} week to evaluate the chemopreventive effects of sinapic acid.

Experimental protocol (In vivo studies)



Genotoxicity study

The male golden Syrian hamsters were divided into 4 groups of 8 each. Group I, served as an untreated control. The Group II and Group III were pretreated with 50mg/kg b.w of sinapic acid for 5 days. At the end of 5th day Group II and III animals were intraperitoneally injected with DMBA (20mg/kg b.w. single dose) after 2hrs administration of sinapic acid. Group IV was orally administrated with sinapic acid to exclude any toxic effects. All animals were sacrificed after 6th day for assessment of chromosomal aberration, frequency of micro-nucleated polychromatic erythrocytes (MnPCE) and detoxification enzymes.

Samples used in the study

- ✓ Blood (Plasma, Erythrocyte, Erythrocyte lysate and Erythrocyte membrane)
- ✓ Tissues (Liver and buccal mucosa)

Blood samples

Blood samples were collected into heparinized tubes. Plasma was separated by centrifugation at 1500 x g for 15 minutes.

Preparation of hemolysate

The erythrocytes remaining after the removal of plasma were washed three times with 310 mM isotonic Tris-HCl buffer (pH 7.4). Hemolysis was carried out by pipetting out the washed erythrocyte suspension into polypropylene centrifuge tubes, which contained 20mM hypotonic Tris-HCl buffer (pH 7.2). The hemolysate was separated by centrifugation at 3500 xg for 15min at 20°C.

Isolation of erythrocyte membrane

The erythrocyte membrane was prepared by the method of Quist, (1980).

Reagents

1. 310 mM isotonic Tris-HCl buffer (pH 7.4).
2. 20 mM hypotonic Tris-HCl buffer (pH 7.2).

Procedure

The packed cells remaining after the removal of plasma were washed thrice with isotonic Tris-HCl buffer, pH 7.4. Hemolysis was performed by pipetting out the washed suspension of red blood cells into polypropylene centrifuge tubes

containing hypotonic Tris-HCl buffer, pH 7.2. Ghosts were sediment by using a high-speed refrigerated centrifuge at 20,000g for 40 minutes. The hemolysate was decanted carefully and the ghost button was re-suspended by swirling. Sufficient buffer of the same strength was added to reconstitute the original volume. The ratio of the cells to washing solution was approximately 1:3 by volume. The same procedure was repeated for three times until membrane become colourless or pale pink. The pellet of erythrocytes was re-suspended in a known volume of 0.1 M Tris-HCl buffer, pH 7.2. Aliquots from this preparation were used for biochemical estimations.

Preparation of tissue homogenate

Tissue samples from animals were washed with ice cold saline and dried between folds of filter paper, weighed and homogenized using appropriate buffer [appropriate buffer of concerned parameter (TBARS-0.025 M Tris-Hcl buffer, pH 7.5; GSH and GPx- 0.4 M phosphate buffer, pH - 7.0; SOD - 0.025 M sodium pyrophosphate buffer, pH 8.3; CAT - 0.01 M phosphate buffer, pH 7.0) in an All glass homogenizer with teflon pestle. The homogenate was centrifuged at 1000g for 5 minutes and the supernatant was then used for the biochemical estimations.

Biochemical and molecular studies

Estimation of lipid peroxidation	:	TBARS
Estimation of enzymatic antioxidants	:	SOD, CAT and GPx.
Estimation of non- enzymatic antioxidants	:	Vit-C, Vit-E and GSH.
Estimation of detoxifying enzyme	:	Cyt p450, Cyt b5, GST, GR.
Glycoconjugates	:	Protein bound hexose hexosamine, total sialic acid, fucose, lipid bound sialic acid.
Lipids and Lipoprotein	:	Total cholesterol, HDL, VLDL, LDL, Phospholipids, Free fatty acids, triglycerides, potassium, sodium and $Na^{+}K^{+}$ ATPase.
Genotoxicity markers	:	MnPCE, CA and Single cell gel electrophoresis (COMET Assay).
Apoptotic markers	:	P^{53}, Bcl-2, BAX, Caspase-3 and TNF-α.

Biochemical and molecular studies were conducted on plasma, lysate, liver homogenate and buccal mucosa of control and experimental animals in each group. The biochemical and molecular markers that were related to carcinogenic process are analysed. The biochemical and molecular parameters that were related to carcinogenic process were analysed.

Biochemical estimations

Biochemical estimations were carried out in blood, liver and buccal mucosa samples of control and experimental animals in each group.

Estimation of lipid peroxidation by products

Estimation of lipid hydroperoxides

Lipid hydroperoxides in the plasma and tissues were estimated by the method of Jiang *et al.,* (1992). Oxidation of ferrous ion (Fe^{2+}) under acidic conditions in the presence of xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560 nm.

Reagents

1. Fox reagent: 88 mg butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium iron (II) sulphate were added to 90 mL methanol and 10 mL H_2SO_4 (250 mM) mixture.

Procedure

0.9 mL Fox reagent was mixed with 0.1 mL of the sample, incubated for 30 min at room temperature and the absorbance was read 560 nm.

Lipid hydroperoxides were expressed as mmol/dL of plasma or mmol/100 g of tissues.

Plasma TBARS

Lipid peroxidation was estimated as evidenced by the formation of TBARS. TBARS in plasma were assayed by the method of Yagi, (1987).

Plasma was de-proteinised with phosphotungstic acid and the precipitate was treated with thiobarbituric acid at 90^{o}C for 1 hour. The pink color formed gives a measure of the thiobarbituric acid reactive substances (TBARS) which was read at 530 nm.

Reagents

1. 0.083 N sulphuric acid
2. 10% phosphotungstic acid
3. Thiobarbituric acid (TBA): 670 mg was dissolved in 100 mL water. To this, 100 mL of glacial acetic acid was added.
4. Standard malondialdehyde stock solution: 1,1',3,3'-tetramethoxy propane (184 μg/mL).

Procedure

To 0.5 mL of plasma, 4.0 mL of 0.083 N sulphuric acid was added. To this mixture, 0.5 mL of 10% phosphotungstic acid was added and mixed. After standing at room temperature for 5 minutes the mixture was centrifuged at 3,000 x g for 10 minutes. The supernatant was discarded. The sediment was mixed with 2.0mL of sulphuric acid and 0.3 mL of 10% phosphotungstic acid. The mixture was shaken well and centrifuged at 3,000g for 10 minutes. The sediment was suspended in 4.0 mL of distilled water and then 1.0 mL of TBA reagent was added. The reaction mixture was heated at 95°C for 60 minutes. After cooling, 5.0 mL of n-butanol was added and the mixture was shaken vigorously and centrifuged at 3000 g for15 minutes. The color extracted in the butanol layer was read at 530 nm. Tubes containing standard malondialdehyde 1 to 5 nmoles were treated in a similar manner along with a blank containing 4.0 mL of distilled water.

TBARS level was expressed as nmol/mL plasma.

Estimation of erythrocyte TBARS

TBARS in erythrocytes and erythrocyte membrane was estimated by the method of Donnan, (1950).

The pink chromogen formed by the reaction of thiobarbituric acid with breakdown products of lipid peroxides was read at 535 nm.

Reagents

1. Stock malondialdehyde solution: 1,1'3,3' tetra methoxy propane (184 μg/mL)
2. 10% trichloroacetic acid (TCA)
3. 0.67% thiobarbituric acid (TBA).

Procedure

The reaction mixture in a total volume of 1.7 mL contained 0.2 mL of erythrocytes/erythrocyte membrane and 1.5 mL of 10% trichloroacetic acid. The mixture was filtered through Whatmann No.1 filter paper. Thiobarbituric acid was added to the portion of filtrate (usually 0.6 mL or 0.8 mL) in the ratio of 1.2:1mL. The mixture was heated in a boiling water bath for 15 minutes, cooled to room temperature and the color developed was measured at 535nm.

TBARS values were expressed as p moles/mg Hb for erythrocytes and nmoles/mg protein for erythrocyte membranes.

Tissue TBARS

Lipid peroxidation (TBARS) in buccal mucosa was estimated by the method of Ohkawa, (1979).

Reagents

1. 8.1% Sodium dodecyl sulphate
2. 20% Glacial acetic acid
3. n-Butanol and Pyridine (5:1 v/v)
4. Standard solution: 1,1',3,3'– tetra methoxy propane 0.5 nmol /mL

Procedure

To 0.2 mL of buccal mucosa tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate and 1.5 mL of 20% acetic acid were added. The pH was adjusted to 3.5 with sodium hydroxide, then 1.5 mL of 0.8% aqueous solution of TBA was added to the mixture and the volume was made up to 4 mL with distilled water. The reaction mixture was heated in an oil bath at 95°C for 60 minutes. After

cooling by tap water, 1 mL of distilled water and 5 mL of n-butanol pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the organic layer was removed and absorbance was read at 535 nm.

The level of TBARS in tissue was expressed as nmol/100 mg protein.

Estimation of enzymatic antioxidants

Estimation of Superoxide Dismutase (SOD)

Superoxide dismutase activity in plasma and buccal mucosa was assayed by the method of Kakkar, (1984).

The assay is based on the inhibition of the formation of NADH-phenazine metho sulphate-nitroblue tetrazolium formazan. The reaction is initiated by the addition of NADH. After incubation for 90 seconds, the reaction is stopped by adding glacial acetic acid. The color developed at the end of the reaction is extracted into n-butanol layer and measured at 520 nm.

Reagents

1. 0.052 M Sodium pyrophosphate buffer (pH 8.3)
2. Absolute ethanol
3. Chloroform
4. n-Butanol
5. 186 μmol Phenazinemethosulphate (PMS)
6. 300 μmol Nitrobluetetrazolium (NBT)
7. 780 μmol Reduced nicotinamide adenine dinucleotide (NADH)

Procedure

0.5 mL of the plasma or buccal mucosa tissue homogenate was diluted to 1.0 mL with ice cold water, followed by 2.5 mL ethanol and 1.5 mL chloroform (chilled reagents). This mixture was shaken for 60 seconds at 4°C and then centrifuged. The enzyme activity in the supernatant was determined as follows.

The assay mixture contained 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of PMS and 0.3 mL of NBT and appropriately diluted enzyme preparation in a total volume of 3 mL. The reaction was started by the addition of 0.2 mL NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1 mL glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 mL n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and the butanol layer was separated. The color intensity of the chromogen in butanol layer was measured in a colorimeter at 520 nm. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration, which gives 50 % inhibition of NBT reduction in one minute under assay conditions.

Superoxide dismutase activity was expressed as U/mL for plasma and U/mg protein for tissue.

Estimation of Catalase

The activity of catalase in plasma and buccal mucosa was assayed by the method of Sinha, (1972).

Dichromate in acetic acid was reduced to chromic acetate, when heated in the presence of hydrogen peroxide with the formation of perchromic acid as an unstable intermediate. The chromic acetate formed was measured at 590 nm. Catalase was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate-acetic acid mixture and the remaining H_2O_2 was determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

Reagents

1. 0.01 M Phosphate buffer (pH 7.0)
2. 0.2 M Hydrogen peroxide
3. 5 % Potassium dichromate
4. Dichromate-acetic acid reagent: Potassium dichromate and glacial acetic acid were mixed in the ratio of 1:3. From this 1 mL was diluted again with 4 mL of acetic acid.
5. 0.2 mM Standard hydrogen peroxide

Procedure

To 0.9 mL phosphate buffer, 0.1 mL plasma or buccal mucosa tissue homogenate and 0.4 mL hydrogen peroxide were added. The reaction was arrested after 15, 30, 45 and 60 seconds by adding 2.0 mL of dichromate-acetic acid mixture. The tubes were kept in a boiling water bath for 10 minutes, cooled and the color developed was read at 530 nm. Standards in the concentration range of 20-100 μmoles were processed as for the test.

The activity of catalase was expressed as U/mL for plasma and U/mg protein for tissue.

Estimation of Glutathione Peroxidase

The activity of glutathione peroxidase in plasma and buccal mucosa was determined by the method of Rotruck *et al.,* (1975) with modifications.

A known amount of enzyme preparation was allowed to react with H_2O_2 in the presence of GSH for a specified time. The GSH content remaining after the reaction was measured by the method of Beutler and Kelley, (1963).

Reagents

1. 0.4 M Phosphate buffer (pH 7.0)
2. 10 mM Sodium azide solution
3. 10 % Trichloroacetic acid (TCA)
4. 0.4 mM Ethylene diamine tetra acetic acid (EDTA)
5. 0.2 mM H_2O_2
6. 2 mM Reduced glutathione (GSH)

Procedure

The reaction mixture in a total volume of 1 mL contained 0.2 mL of phosphate buffer, 0.2 mL EDTA, 0.1 mL of sodium azide and 0.5 mL of the enzyme preparation (plasma/tissue homogenate). 0.2mL of glutathione and 0.1 mL of H_2O_2 were added, to this mixture and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 mL of 10 % TCA. The tubes were

centrifuged and the supernatant was assayed for GSH. A blank was treated similarly to which 0.2 mL of the enzyme was added after the incubation.

The activity of glutathione peroxidase was expressed as U/L for plasma and U/g protein for tissues.

Estimation of Non-enzymatic antioxidants

Estimation of Reduced Glutathione (GSH)

The reduced glutathione level in plasma, liver and buccal mucosa was determined by the method of Beutler and Kelley (1963).

This method was based on development of yellow color when 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB) is added to compound containing sulphydryl groups. The color developed was read at 412 nm.

Reagents

1. 0.3M Disodium hydrogen phosphate
2. 0.1% Disodium salt of EDTA
3. Precipitating reagent: 1.67 g of metaphosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in one liter of distilled water.
4. 5'-Dithio (bis)-2-nitrobenzoic acid (DTNB) reagent: 40 mg of DTNB in 100 mL of 1% sodium citrate.
5. Standard solution: 10 mg of reduced glutathione in 100 mL distilled water.

Procedure

0.2 mL of sample (plasma or liver or buccalmocosa homogenate) was mixed with 1.8 mL of EDTA solution. To this 3.0 mL of precipitating reagent was added, mixed thoroughly and kept for 5 minutes before centrifugation. To 2.0 mL of the filtrate, 4.0 mL of 0.3 M disodium hydrogen phosphate solution and 1.0 mL of DTNB reagent were added and the color developed was read at 412 nm. A set of standard solutions containing 20-100 μg of reduced glutathione was treated similarly.

The values were expressed as mg/dl for plasma and mg/100 mg tissue for buccal mucosa.

Estimation of Vitamin C

The level of plasma vitamin C was determined by the method of Omaye *et al.,* (1979).

Ascorbic acid is oxidized by copper to form dehydro ascorbic acid and diketoglutaric acid. These products when treated with 2,4-dinitrophenyl hydrazine (DNPH) form the derivatives bis-2,4-dinitrophenylhydrazone which undergoes rearrangement to form a product with an absorption maximum at 520 nm. Thiourea provides a mild reducing medium that helps to prevent interference from non-ascorbic acid chromogens.

Reagents

1. 2,4-dinitrophenylhydrazine-thiourea - copper sulphate reagent (DTC): 0.4 g thiourea, 0.05 g copper sulphate and 3.0 g of DNPH in 100 mL of 9N H_2SO_4.
2. 10% TCA
3. 65% H_2SO_4
4. Standard solution: 10 mg/100mL of 5% TCA.

Procedure

1.0 mL of the sample was mixed thoroughly with 1.0 mL of ice cold 10% TCA and centrifuged for 20 minutes at 3500 xg. To 0.5 mL of the supernatant, 0.1 mL of DTC reagent was added and mixed well. The tubes were incubated at 37^{o}C for three hours. 0.75 mL of ice cold 65% sulphuric acid was added and the tubes were allowed to stand at room temperature for an additional 30 minutes. A set of standards containing 10-50 μg of ascorbic acid was processed similarly along with a blank containing 0.5 mL of 10% TCA. The colour developed was read at 520nm.

Vitamin C values were expressed as mg/dl for plasma.

Estimation of Vitamin E

Plasma Vitamin E

Vitamin E was estimated in plasma by the method of Palan *et al.,* (1991).

This method involves reduction of ferric ions to ferrous ions by the tocopherol and the formation of a pink coloured complex with batho phenanthroline orthophosphoric acid. Absorbance of the stable chromophore is measured at 536nm.

Reagents

1. Redistilled ethanol
2. Petroleum ether (60-80^{o}C)
3. Bathophenanthroline reagent: 0.2% solution of 4,7-dipyridyl-1-10-phenanthroline in purified absolute ethanol.
4. 0.01M Ferric chloride in absolute ethanol.
5. 0.001M Orthophosphoric acid in absolute ethanol.
6. Standard solution: 1g/100 mL alpha-tocopherol in absolute ethanol.

Procedure

To 0.2 mL of the plasma sample taken in a glass stoppered centrifuged tube, 1.8mL of redistilled ethanol was added and thoroughly mixed. 3.0 mL of petroleum ether was then added and tubes were shaken rapidly in a mechanical shaker for 3 minutes. The tubes were centrifuged and 2.0 mL of the ether layer transferred to fresh tubes and evaporated to dryness. To the lipid residue, carefully re-dissolved in 3.0 mL absolute ethanol, 0.2 mL of 0.2% bathophenanthroline reagent was added and mixed. The tubes were protected from exposure to direct light and the assay was carried out rapidly from this point. 0.2 mL of ferric chloride reagent was added and mixed in a vortex mixture. After one minute, 0.2 mL of orthophosphoric acid reagent was added and shaken well. Tubes containing standard alpha-tocopherol, 2-10 µg was treated in a similar manner along with a blank containing 3.0mL of ethanol. The alpha-tocopherol values were expressed as mg/dl for plasma.

Tissue Vitamin E

The concentration of total vitamin E in buccal mucosa was estimated by the method of Desai, (1984).

Reagents

1. 66% ethanol
2. 60% H_2SO_4
3. Hexane
4. Liquid nitrogen

Procedure

The lipid extracts were dried under nitrogen gas and the residues were suspended in 66% ethanol and then 4 mL of hexane and 0.6 mL of 60% H_2SO_4 were added. The tubes were vortexes and centrifuged. The upper hexane phase was removed its fluorescence intensity was measured at an excitation of 295 nm and emission of 320 nm. The standard alpha-tocopherol was used to prepare the standard graph.

The alpha-tocopherol values were expressed as mg/100 mg for tissue.

Estimation of drug metabolizing enzymes

Preparation of microsomes

Liver and buccal mucosa were minced into small pieces, homogenized in buffer (50 mM Tris-HCl, pH 7.4, 1.15% KCl) and centrifuged at 10,000 xg for 20 minutes. The supernatant was collected and ultra-centrifuged at 100,000 xg for 60 minutes. The supernatant was removed and the microsomal pellet was re-suspended in buffer and re-centrifuged as above. The microsomal pellet was used for the assay of phase-I detoxification enzymes, cytochrome P^{450} and cytochrome b_5.

Estimation of Cytochrome P^{450}

Cytochrome P^{450} in liver and buccal mucosa tissues microsomes was estimated by the method of Omura and Sato, (1964).

Reagents

1. 0.1M Phosphate buffer (pH 7.0)
2. Sodium dithionate
3. Saturated solution of carbon monoxide

Procedure

Microsomes suspended in phosphate buffer (4 mg protein/mL) were reduced by the addition of solid sodium dithionate. Then 1mL of saturated carbon monoxide was added. The absorbance of the samples was read at 450 nm.

The level of cytochrome P^{450} was expressed as µmole/mg protein.

Estimation of Cytochrome b_5

The amount of cytochrome b_5 in liver and buccal mucosa tissues microsomes was measured by the method of Omura and Sato (1964).

Reagents

1. 0.1 M Phosphate buffer (pH 7.0)
2. 0.4 mM NADH

Procedure

To the microsomal suspension, containing 4 mg of protein/mL in phosphate buffer, 1 mL of NADH was added. The absorbance at 450 nm was read against the blank containing microsomal suspension alone.

The level of cytochrome b_5 was expressed as µmole/mg protein.

Estimation of xenobiotic metabolizing enzymes

Estimation of Glutathione-S-Transferase

The activity of GST was assayed by the method of Habig *et al.,* (1974).

GST activity was measured by following the increase in absorbance at 340 nm using 1-chloro 2,4-dinitrobenzene (CDNB) as the substrate.

Reagents

1. 0.3 M Phosphate buffer (pH 6.5)
2. 30 mM GSH
3. 30 mM CDNB in 95% ethanol

Procedure

1.0mL of phosphate buffer, 0.1 mL of CDNB and 0.1 mL of tissue homogenate (liver/ buccal mucosa) was taken in a test tube. The volume was adjusted to 2.9 mL with water. The reaction mixture was pre-incubated at 37°C for 5 minutes and the reaction started by the addition of 0.1 mL of 30 mM glutathione. The absorbance was followed for 5 minutes at 540 nm. A system devoid of enzyme served as the blank.

The specific activity of GST was expressed as nm of CDNB conjugate formed/min/mg protein.

Estimation of Glutathione Reductase

Glutathione reductase activity was assayed in liver and buccal mucosa by the method of Carlberg, (1985).

The enzyme activity was assayed by measuring the GSH formed when the oxidized glutathione (GSSG) is reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Reagents

1. 0.1 M Phosphate buffer (pH 7.4)
2. 0.1 M Sodium bicarbonate solution
3. 250 µM Oxidized glutathione (GSSG)
4. 250 Mm Flavin adenine dinucleotide (FAD)
5. 4 mM Reduced nicotinamide adenine dinucleotide phosphate (NADPH)
6. 80 mM Ethylene diamine tetra acetic acid (EDTA)

Procedure

2.0 mL phosphate buffer, 0.1 mL enzyme sample (liver/buccal mucosa tissue homogenate), 0.1 mL FAD and 0.5 mL EDTA solution were taken in a test tube. A blank was set up using all the reagents except FAD. The tubes were incubated at 37˚C for 15 minutes followed by the addition of 0.1 mL of NADPH solution to each tube. The reaction rate was then continuously monitored at 340 nm for 5 minutes and the linear absorbance change was measured.

Values are expressed in nm of NADPH oxidized/min/mg protein.

Estimation of γ-glutamyl transpeptidase (GGT)

The activity of GGT was assayed by the method of Fiala (1980) using γ-glutamyl-p-nitroanilide as the substrate.

Reagents

1. γ-glutamyl-p-nitroanilide
2. Glycylglycine
3. Tris-Hcl buffer 0.1M (pH 8.0)

Procedure

The reaction mixture containing 22 μmol eglycylglycine, 5μmoles γ-glutamyl-p-nitroanilide and 3μmoles of Tris-HCl buffer (pH 8.0) in a total volume of 4 ml was incubated for 10 minutes at 37^0C. The reaction was arrested by immersing the tubes in a boiling water bath and the absorbance of liberated P-nitroaniline was read at 410nm.

Enzyme activity is expressed as μmoles of P-nitroaniline formed/hr/mg protein

Estimation of glycoconjugates profile

Extraction of glycoproteins

The precipitate obtained after treating the plasma with 95% ethanol was used for the estimation of protein bound hexose and hexosamine. Similarly, the precipitate obtained after treating the erythrocyte membranes with 1%

phosphotungstic acid followed by 5% TCA was used for the estimation of protein bound hexose and hexosamine.

The defatted buccal mucosa tissues obtained after treating the tissues with methanol and chloroform was used for the estimation of glycoproteins. To the dry defatted tissues, remaining after lipid extraction, 0.1 N H_2SO_4 was added and hydrolyzed at 80°C for 1 h. It was cooled and the aliquot was used for sialic acid estimation. To the remaining solution of 0.1 N NaOH was added and kept in an ice bath for 1 h. From these aliquots, protein bound hexose, hexosamine and fucose were estimated.

Estimation of protein bound hexose

The protein bound hexose in plasma and defatted buccal mucosa tissues were estimated by the method of Niebes, (1972). To the glycoprotein extract, orcinol and sulphuric reagent were added and heated for 15 minutes at 80°C. The color developed was read at 540 nm.

Reagents

1. 5% phenol
2. Concentrated sulphuric acid
3. Galactose-mannose standard: 0.2 mg/mL and 0.1 mg/mL each of galactose and mannose.

Procedure

0.5 mL of aliquot was mixed with 0.5 mL 5% phenol and 2 mL concentrated sulphuric acid were added. 0.5 mL of 0.1 N NaOH for blank and standards in the concentration range of 40-200 µg were treated in the similar way. The tubes were then heated in a boiling water bath for 20 minutes and the absorbance was measured at 490 nm. The concentration of protein bound hexose was expressed as mg/dl for plasma and mg/g protein for defatted tissue.

Estimation of protein bound hexosamine

The protein bound hexosamine in plasma was estimated by the method of Wagner, (1979).

Reagents

1. 3.5% Acetylacetone reagent: the reagent was prepared by mixing 98 mL of 1N trisodium phosphate and 2 mL of 0.5N potassium tetraborate.
2. Ehrlich's reagent: 320 mg of p-dimethyl amino benzaldehyde was dissolved in 20 mL of isopropanol and 3.0 mL of concentrated HCl.
3. 3 N HCl
4. 6 N HCl
5. Standard galactosamine: 10 mg of galactosamine hydrocholoride was dissolved in 100 mL of water (100 µg/mL).

Procedure

0.1 mL of extract was treated with 2.5 mL of 3 N HCl for 6 hours in a boiling water bath and then neutralized with 6 N NaOH. To 0.8 mL of neutralized sample, added 0.6 mL of acetylacetone reagent. The tubes were heated in a boiling water bath for 30 minutes. After cooling 2 mL of Ehrlich's reagent was added and mixed well. Blank contained 1.0 mL NaOH was treated in the same way. The color developed was read at 540 nm.

The concentration of protein bound hexosamine was expressed as mg/dl for plasma.

Estimation of sialic acid

The total sialic acid in plasma and defatted buccal mucosa tissues were estimated by the method of Warren, (1959).

Reagents

1. 0.025 M Periodic acid in 0.1 N sulphuric acid
2. 4% Sodium meta arsenate in 0.5 N HCl
3. Thiobarbituric acid: 144 mg of thiobarbituric acid dissolved in 10 mL hot distilled water
4. Acidified butanol: 5 % HCl in n-butanol
5. Standard sialic acid: 179 mg of orosomucoid was dissolved in 100 mL of distilled water (0.2 mg/mL)

Procedure

To 0.5 mL of sample (plasma/defatted buccal mucosa tissues) in a test tube, 0.5 mL of water and 0.25 mL of periodic acid were added and incubated at 37˚C for 30 minutes. To this 0.25 mL of sodium m-arsenate and 2 mL of TBA were added and heated in a boiling water bath for 6 minutes. It was cooled and 5 mL of acidified butanol was added. The absorbance by the extract in the organic layer was read at 540 nm against the reagent blank.

The concentration of total sialic acid was expressed as mg/dl for plasma and mg/g protein for defatted tissue.

Estimation of lipid bound sialic acid

Plasma lipid bound sialic acid concentration was determined by the procedure of Katopodis and Stock, (1980).

Reagents

1. chloroform: methanol 2:1 (v/v)
2. phosphotungstic acid
3. Standard sialic acid: 179 mg of orosomucoid was dissolved in 100mL of distilled water (0.2 mg/mL)

Procedure

0.05 mL of plasma was extracted with 3.0 mL of chloroform: methanol 2:1 (v/v) at 4 to 5°C. The lipid extract was partitioned with 0.5 mL of cold distilled water and the aqueous phase containing the sialo-lipid fraction was precipitated with 0.05 mL of phosphotungstic acid solution (10 mL). After centrifugation, the supernatant fluid was aspirated, the precipitate was re-suspended in 1 mL of distilled water, and sialic acid in suspension was determined by TBA method.

Lipid bound sialic acid was expressed as mg/dl for plasma.

Estimation of fucose

Fucose in plasma and buccal mucosa tissue were estimated by the method of Dische and Shettles (1948).

Plasma was heating with H_2SO_4 for 10 minutes followed by the addition of cysteine hydrochloride. The absorbance of the color developed was read at 430 nm.

Reagents

1. 95 % Ethanol
2. 0.1 N Sodium hydroxide
3. 6:1 (v/v) Sulphuric acid- water mixture
4. Cysteine hydrochloride reagent: 3 g in 100mL distilled water
5. Stock standard: 50 mg fucose was dissolved in 100 mL of distilled water
6. Working standard: 10 mL of stock standard was diluted to 100 mL with distilled water to get a concentration of 50 µg/mL

Procedure

To 0.5 mL of aliquot, 4.5 mL of sulphuric acid-water mixture was added. The tubes were kept in boiling water bath for 3 minutes and cooled. 0.1 mL of cysteine hydrochloride reagent and 0.5 mL of 0.1 N NaOH were added. Blank and standards in the concentration range of 5-25 µg were also treated same way. After 75 minutes in dark, absorbance was noted at 430nm. The fucose concentration was expressed as mg/dl plasma and mg/g protein for defatted tissue.

Estimation of lipids

Extraction of lipids

Lipid extraction was done by the method of Folch *et al.,* (1957).

2.0 mL of plasma or 2.5 mL of erythrocyte membrane or 500 mg tissue was homogenized in 7.0 mL of methanol. The contents were filtered in Whatmann No.1 filter paper and the residue on the filter paper was scrapped off and homogenized with 14 mL of chloroform. This was filtered and the residue was successively homogenized in chloroform-methanol (2:1v/v) and each time the extract was filtered. The pooled filtrates in the filter flask were adjusted to a final volume of 5.0 mL with chloroform-methanol mixture (2:1 v/v) and evaporated to

dryness to a constant weight. The weights of the flasks with dried lipids were recorded. The dried residue was dissolved in 5.0 mL of chloroform-methanol mixture (2:1 v/v) and transferred to a tube.To the re-dissolved lipid extract, 1.0 mL of 0.1 M potassium chloride was added, shaken well and centrifuged. The upper aqueous layer containing gangliosides was discarded. The chloroform layer was mixed with 1.0 mL of chloroform-methanol-potassium chloride (1:10:10 v/v) mixture and then centrifuged. The washing was repeated thrice and each time the upper layer was discarded. The lower layer was made upto a known volume and used for the analysis of lipids.

Estimation of total cholesterol

Total cholesterol was estimated by the method of Parek and Jung, (1970).

Reagents

1. Ferric chloride–uranyl acetate reagent: 10 mL of water and 3 mL of concentrated ammonia was added to 500 mg of ferric chloride. The precipitate obtained was washed several times with distilled water, dissolved in glacial acetic acid and made upto 1 litre with acetic acid. 100 mg of uranyl acetate was then added and the contents were shaken well and left overnight in a brown bottle.
2. Ferrous sulphate and sulphuric acid reagent: 100 mg of anhydrous ferrous sulphate in 100 mL of glacial acetic acid was made upto 1 litre with concentrated sulphuric acid.
3. Cholesterol standard: 2 mg/mL chloroform.

Procedure

A known volume of the lipid extract was added to 10 mL of ferric acetate-uranyl acetate reagent. The mixture was kept for 5 minutes and centrifuged at 1000 g for 5 minutes and 2 mL of ferrous sulphate-sulphuric acid reagent was added to 3.0mL aliquot of the supernatant. After 20 minutes, the colour was read at 540 nm along with a series of standard cholesterol solutions (25-100 μg) and a blank containing the reagent processed in a similar manner.

Total cholesterol values were expressed as mg/dl for plasma and μg/mg protein for erythrocyte membrane and mg/g tissue for tissues.

Estimation of phospholipids

Phospholipids were estimated in plasma and erythrocyte membrane by the method of Zilversmit and Davis, (1950).

The organic phospholipid phosphours is converted to inorganic phosphorus, which reacts with ammonium molybdate to form phosphomolybdic acid. This on reduction with aminonaphtholsulfonic acid forms a stable blue color, which was read at 680 nm.

Reagents

1. Concentrated sulphuric acid
2. Concentrated nitric acid
3. Ammonium molybdate: 2.5% in 5 N H_2SO_4
4. 1-amino 2-naphthol 4-sulphonic acid (ANSA):500 mg ANSA was dissolved in a mixture of 195 mL 15% sodium bisulphate and 5 mL 20% sodium sulphite solution. This solution was stored in a brown bottle.
5. Standard phosphorus solution: 35.1 mg of potassium dihydrogen phosphate was dissolved in water. To this 1 mL of 10 N sulphuric acid was added and made upto 100 mL with distilled water. 10mL of this solution was diluted to 100 mL, to prepare a working standard containing 8 μg phosphorus/mL.

Procedure

0.1 mL of plasma was mixed with 1.9 mL of distilled water and 1.5 mL of 10% TCA. The precipitated proteins were sedimented by centrifugation. The supernatant was discarded. 1 mL of concentrated nitric acid and 1 mL of concentrated sulphuric acid was added to the residue and digested on a sand bath till the solution become colourless.

In erythrocyte membrane/tissue homogenate a known amount of the lipid extract was digested with 1.0 mL of 70% perchloric acid over a sand bath until the sample become colourless and clear. After cooling, the volume was made upto 5 mL with water. Standard solutions of phosphorus in the range of 8-32 μg and a

blank with double distilled water were also prepared. Then 1 mL of ammonium molybdate solution was added, followed by 0.4 mL of ANSA reagent. The absorbance was read at 680nm after 5 minutes. Phospholipid contents were calculated after multiplication by a factor 25 of the obtained phosphorus concentration.

Phospholipid level was expressed as mg/dl for plasma and μg/mg protein for erythrocyte membrane and mg/g tissue for tissues.

Estimation of free fatty acids

Free Fatty Acids were estimated by the method of Falholf *et al.,* (1973).

The method is based on copper soap formation. In the presence of phosphate buffer, the extract was taken with a high-density copper reagent and the copper soap remained in the upper organic layer, was determined calorimetrically with diphenylcarbazide at 550nm.

Reagents

1. Extraction solvent: Chloroform: Heptane: Methanol (5:5:1).
2. Phosphate buffer (pH6.4): 4.539 g/l potassium dihydrogen phosphate and 5.939 g/l disodium hydrogen phosphate (2:1v/v).
3. Stock copper solution: 50 mM copper nitrate/litre
4. 1 M Triethanolamine.
5. 1 M Sodium hydroxide.
6. Copper reagent: 10 mL of copper solution, 10 mL of triethanolamine and 6 mL of sodium hydroxide were mixed well and diluted to 100mL with water. To this, 33 g of sodium chloride was added and the pH was adjusted to 8.1.
7. Diphenylcarbazide solution: 40 mg in 10 mL ethanol with 0.1 mL triethanolamine.
8. Palmitic acid: 2 mM/litre.

Procedure

A known volume of sample was evaporated to dryness to which 1.0 mL phosphate buffer, 6.0 mL extraction solvent and 2.5 mL copper reagent were added. The blank and standard (10-50µg) were also treated in the same manner. The tubes were shaken vigorously for 90 seconds, allowed to stand for 15 minutes and centrifuged at 4000 xg for 5 minutes. 3.0mL of the upper layer was transferred to a tube containing 0.5 mL diphenylcarbazide solution, mixed carefully and read after 15 minutes at 550nm.

Free fatty acids were expressed as mg/dl for plasma and mg/g tissue for tissues.

Estimation of triglycerides

Triglycerides were estimated by the method of Foster and Dunn, (1973).

The triglyceride was extracted by isopropanol, which upon saponification with potassium hydroxide yield glycerol and soap. The glycerol liberated is treated with metaperiodate, which release formaldehyde, formic acid and iodide. The formaldehyde released reacts with acetylacetone and ammonia forming yellow colored compound, which was read at 405nm.

Reagents

1. Isopropanol
2. Activated alumina (neutral)
3. Saponification agent: 5.0 g of potassium hydroxide were dissolved in 60 mL distilled water and 40 mL isopropanol was added to it.
4. Acetyl acetone reagent: 0.75 mL of acetyl acetone was dissolved in 60 mL distilled water and 40 mL isopropanol was added to it.
5. Sodium metaperiodate reagent: 77 g of anhydrous ammonium acetate was dissolved in about 700 mL of distilled water; 60 mL glacial acetic acid was added to it followed by 650 mg sodium metaperiodate. The mixture was dissolved and diluted to one litre with distilled water.

6. Standard solution of triolein: 1.0 g of triolein was dissolved in 100 mL isopropanol. 1.0 mL of stock standard was diluted to 100 mL to prepare working standard containing 100 μg of triolein/mL

Procedure

An aliquot (0.5 mL) of lipid extract was evaporated to dryness. Added 0.1 mL of methanol followed by 4.0 mL of isopropanol. About 0.4 g of alumina was added to all the tubes and shaken well for 15 minutes. Centrifuged and then 2.0 mL of the supernatant was transferred to appropriately labelled tubes. The tubes were placed in a water bath at 65°C for 15 minutes for saponification after adding 0.6 mL of the saponification reagent followed by 0.5 mL of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65°C for an hour. A series of standards of concentration 8-40 μg triolein were treated similarly along with a blank containing only the reagents. All the tubes were cooled and read at 405 nm.

The amount of triglycerides was expressed as mg/dl for plasma.

Estimation of erythrocyte membrane Na^+/K^+-ATPase

Na^+/K^+-ATPase was assayed in erythrocyte membrane according to the procedure of Bonting, (1970).

Reagents

1. 184 mM Tris-HCl buffer (pH 7.5)
2. 50 mM magnesium sulphate
3. 50 mM potassium chloride
4. 600 mM sodium chloride
5. 1 mM EDTA
6. 40 mM ATP
7. 10 % trichloroacetic acid
8. Fiske and Subbarow reagents

Procedure

The incubation mixture contained 1.0 mL of buffer, 0.2 mL of magnesium sulphate, 0.2 mL of potassium chloride, 0.2 mL of sodium chloride, 0.2 mL of EDTA and 0.2 mL of ATP. After incubation at 37°C, for 10min the reaction was initiated by the addition of 0.2 mL erythrocyte membrane. The contents were incubated at 37°C for 15min. 1.0 mL of 10% TCA was added at the end of 15min to arrest the reaction.

The reaction mixture in a total volume of 10 mL contained 8.0 mL of 10% TCA and 2.0 mL of the supernatant. After 5minutes, the mixture was centrifuged and 5.0 mL of the filtrate was added to 1.0 mL of molybdate solution II (2.5% ammonium molybdate in 3 N H_2SO_4) followed by 0.4 mL of 0.25% ANSA (0.5 g in ANSA in 195 mL of 15% sodium bisulphate and 5 mL of 20% sodium sulphite), and made up to 10mL with distilled water and read at 680nm after 5 minutes. A series of standard phosphorus solution (4-32 µg) were treated similarly.

The enzyme activity in erythrocyte membrane was expressed as µ moles of inorganic phosphorous liberated/h/mg protein.

Cytogenetic Parameters

Bone marrow micronucleus assay

The bone marrow micronucleus assay was carried out according to the method of Schmid, (1975).

The *in vivo* mammalian micronucleus test provides information about chromosome breakage, spindle dysfunction and mitotic non-dysjunction of whole chromosomes. Polychromatic erythrocytes (PCE) in bone marrow of rodents are used in this assay. When the erythroblast develops into an erythrocyte, the main nucleus is extruded and may leave a micronucleus in the cytoplasm, which arise mainly from chromosomal fragments that are not incorporated into the daughter nucleus at the time of cell division. The visualization of the micronucleus is facilitated in the PCEs, because they lack main nucleus. The assay is based on an increase or decrease in the frequency of micro nucleated PCEs (MnPCEs) in bone marrow of the treated animals.

Reagents

1. Fetal calf serum
2. May-Grunwald stain: 250 mg of the May-Grunwald stain powder was dissolved in 100 mL of methanol and was mixed with a magnetic stirrer for 10-15 minutes. It was then filtered twice through Whatman No.1 filter paper. The stain was prepared fresh before use.
3. Giemsa stain: One gram of Giemsa powder was dissolved in 56 mL of glycerol and stirred using a magnetic stirrer at 50ºC overnight. After cooling to room temperature, 84 mL of methanol was added and mixed for 1 h. This solution was filtered through Whatman No.1 filter paper and stored in dark bottle at 4ºC.

Slides

The slides were immersed in 10% chromic acid overnight. They were rinsed in tap water and soaked in soap solution for 1h. The slides were then scrubbed well and left in running tap water for 15 min. They were then rinsed twice in distilled water and blotted using a coarse filter paper. They were then cleaned with methanol and stored in slide boxes.

Procedure

After the experimental period, all the animals were sacrificed by cervical dislocation. The femur bones were removed and cleaned using a coarse filter paper. The kneecap was gently opened using scissors and the contents of the femur bones were flushed into 2 mL of fetal calf serum. It was gently aspirated to give a homogeneous suspension. This suspension was centrifuged at 2000 rpm for 10 min and the supernatant was removed. The pellet was re-suspended in a drop of fresh serum before being used for preparing slides. The slides were then air-dried for 18 h. For each animal three slides were prepared.

Staining

The air-dried slides were stained with May-Grunwald and Giemsa solution as described by Schmid, (1975). The slides were initially stained in undiluted May-Grunwald for 3 min, followed by staining in diluted May-Grunwald stain (1:1

dilution with water) for 2 min and rinsed in water. Then the slides were finally stained in Giemsa (1:6 dilutions with distilled water) for 5-7 min, rinsed in double distilled water and blotted using a filter paper.

Analysis

The permanent slides were coded before staining. For each experimental animal, 2500 polychromatic erythrocytes (PCEs) with or without micronuclei and the corresponding NCEs were scored. PCE's stain bluish purple colour and NCE's stain yellowish orange colour. Micronuclei are round bodies, with a diameter of $1/20^{th}$ -$1/5^{th}$ of an erythrocyte. They stain intensively, similar to the staining of the main nuclei in the nucleated cells. They appear as a spot in the periphery of the cell.

Bone marrow chromosomal aberration analysis

Bone marrow preparations for chromosomal aberration analysis were made according to the procedure of Killian, (1977).

The mammalian *in vivo* chromosomal aberration analysis is used for the detection of structural chromosomal aberrations induced by test compounds in the bone marrow cells of animals. This chromosomal aberration test is especially relevant for assessing mutagenic hazards, in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and DNA-repair processes, although these may vary among species and tissues. Structural aberrations are detectable by microscopic examination of the metaphase of cell division, observed as deletions, fragments, interchanges or interchanges.

Chromatid-type aberration: Structural chromosome damage expressed as breakage of single chromatid or breakage and reunion between chromatids.

Chromosome-type aberration: Structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Gap: An achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of chromatid(s).

Reagents

1. 0.01 % Colchicine in double distilled water.
2. 0.9 % Nacl (saline).
3. Hypotonic potassium chloride solution (0.075 M).
4. Phosphate buffer: 10% Disodium hydrogen phosphate.
5. Methanol: acetic acid (3:1)

Procedure

All the experimental animals were injected intraperitoneally with 1mL of colchicine 90 min prior to sacrifice. Animals were sacrificed by cervical dislocation and the femur bones were removed, cleaned and cut at both ends. Bone marrow cells were flushed out into 5 mL of 0.9% NaCl. The contents were centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the cells were re-suspended in 6 mL of 0.075 M Kcl and incubated at 37ºC for 25 min. Following this, the cells were pelleted and fixed by adding freshly prepared fixative (methanol: acetic acid (3:1)) Fixation was repeated twice. Just prior to slide preparation, the contents in the third fixative were spun, supernatant was discarded and the cell button was suspended in a small quantity (0.5-0.7 mL) of fresh fixative.

Slide preparation

Slides were cleaned in chromic acid and soap solution, washed well and stored in distilled water in the refrigerator prior to use. A test slide was prepared by putting a drop of the cell suspension in fixative on a cold - damp slide. It was gently blown and allowed to dry on a slide warmer. The slide was examined for the optimal number of cells in a given area of the slide. Based on this, necessary dilutions were made to get the optimal concentration of cells.

Staining

Four parts of Giemsa stock solution was added to four parts of 10% Na_2HPO_4 buffer solution and forty two parts of distilled water. The slides were stained for 5-8 min in Giemsa stain and washed for 1-2 min in water and air-dried and examined.

Analysis

All slides were independently coded before staining. Hundred well spread metaphase cells were analyzed for each animal and structural chromosome aberrations in the form of gaps, breaks, acentric fragments, etc were visualized and recorded.

COMET assay (Single-cell gel electrophoresis)

The DNA damage in the bone marrow cells of male golden Syrian hamsters was assessed by the method of Tice *et al.,* (2000).

The comet assay (single-cell gel electrophoresis) is a rapid, simple and reliable technique used to assess the DNA damage in bone marrow cells. Cells embedded in agarose on a microscope slide are lysed with detergent and high salt to form nucleoids containing super coiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks. The likely basis for this is that loops containing a break lose their super coiling and become free to extend toward the anode.

Requirements

1. 1% Normal melting point agarose (NMPA)
2. 0.5% Low melting point agarose (LMPA)
3. Lysing solution
4. 20 μg/mL Ethidium bromide
5. Alkaline electrophoresis buffer (pH>13)

Procedure

The femur bone marrow cells were flushed into Hank's balanced salt solution (HBSS) and then filtered through a 50 μm nylon filter. The cells were counted and diluted to arrive a final suspension of 50,000 - 1,00,000 cells/mL. The mixture of 10 μl bone marrow cells and 200 μl of 0.5% low melting point agarose were layered onto a pre-coated slide which contains 1% normal melting point

agarose and then covered with a cover slip. The slides were placed in the chilled lysing solution containing 2.5 M Nacl, 100 mM Na^{2+} EDTA, 100 mM Tris-HCL, pH 10 and 1% DMSO, 1% Triton X 100 and 1% sodium sarcosinate for 1 h at 4˚C and followed by alkaline buffer (10 N NaOH, 0.5M EDTA and DMSO; pH>13) for 20 minutes. The electrophoresis was carried out for 20 minutes, at 25V (~0.74 V/Cm) and 300 mA. The slides were stained with 50 µl of ethidium bromide (EtBr, 20 µg/mL) and analyzed under 40x objectives on a fluorescence microscope and images (50 cells/sample) were viewed under high performance Nikon camera (Nikon coolpix 4500).

Analysis

DNA damage, as reflected by %DNA in tail (tail intensity), tail length, tail moment (product of tail DNA/ total DNA by the centre of gravity) and olive tail moment (the product of the distance between the barycentre of the head and tail and the proportion of DNA in the tail) of the stored images, was investigated from 50 cells per treatment using CASP software (http://casp.sourceforge.net).

Histopathological staining

Histological slides were prepared by according to the method of Ramos Vara, (2005). For histopathological studies, tumor and normal buccal mucosa were fixed in 10% neutral buffered formalin for 24 hours and were routinely processed and paraffin embedded, 5µm sections were cut in a rotary microtome and mounted on clean glass slides, The Sections of tissues were incubated at 60°C for 1 hour in a hot plate and allowed to reach room temperature and then the samples were immersed in xylene for 1 to 2 minutes to dissolve the wax. The sections were hydrated through descending grades of alcohol (100%, 90%, 70% and 50%) to water and stained in Harri's haematoxylin for 3 minutes followed by washing in running tap water for 10 minutes (Blueing). The sections tissues were differentiated in 1% acid alcohol and stained with eosin for a minute. Then the sections were dehydrated through ascending grades of alcohol (50%, 70%, 90% and 100%) cleared in xylene and mounted with resinous medium (DPX). Finally, it is used to histological studies for evaluate the histopathological changes.

Immunohistochemical staining

To examine the expression patterns and inter correlation of apoptosis related molecular markers p53, bcl-2, Bax, and caspase-3 by immunohistochemical staining methods. 5µm paraffin embedded tissue sections were dewaxed in xylene and hydrated with graded ethanol. The pretreatment was performed in tris citrate buffer (pH 6.0) at 98°C for 40 min. After the pretreatment the slides were stained by horseradish peroxidase (HRP) polymer standard method. The primary antibodies of anti-mouse p53, anti-mouse bcl-2, anti-mouse caspase-3 and anti-mouse Bax were diluted 1:100 and incubated at room temperature in a humidity chamber for 30 min. All the reactions were performed manually at room temperature. Immunolabelling was visualized by inhibition in 3,3-Diaminobenzidine (DAB) solution used as the chromogenic substrate and the tissue sections were counterstained with hematoxylin and permentently mounted.

Statistical analysis

The values are expressed as mean ± SD. The statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT), using SPSS version 12.0 for windows (SPSS Inc. Chicago; http://www.spss.com). The values are considered statistically significant if the *p*- value was less than 0.05.