Materials and methods

Materials

HSA was purchased from Sigma [A-1887, 96% pure, fatty acid free] and used without further purification. p-Benzoquinone (p-BQ) was obtained from Himedia [RM-489] and freshly recrystallized from n-hexane before use. Amino acids were from L-Amino acid Kit Himedia [RM 4020]. Nα-Acetyl Lysine (A2010), Nε-Acetyl Lysine (A4021), N- Acetyl Tryptophan (A6376), N-Acetyl L-Alanine (A4625), Nα-Acetyl L-Arginine (A3133), 8-Anilino-1-naphthalenesulfonic acid (A1028), Quercetin dehydrate (Q0125), Myristic acid (M3128) and Fluorescamine (F9015) were from Sigma Aldrich. Paracetamol (99-100%) was a gift from GlaxoSmithKline Pharmaceuticals Limited, India. N-Acetyl L-Cysteine (0148276) was from Sisco Research Laboratories (SRL). Vitamin C purified was from Merck. All other reagents used were of AR grade. Custom made peptides were obtained from USV Limited. All the spectrofluorimetric analyses were carried out in a Hitachi F-7000 spectrofluorimeter using a 10 mm or 5 mm path length (as needed) quartz cell. Absorbance spectra were taken in a Shimadzu UV-2540 spectrophotometer.

Ethics Statement

All methods were approved by the Institutional Animal Ethics Committee, Permit No. 797/CPCSEA, University of Calcutta. All efforts were made to minimize suffering of the animals. The collection of blood and subsequent experiments with human serum were approved by the Institutional Bioethics Committee for animal and human research studies, University of Calcutta, permission No. 1096, following the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Collection of blood from human volunteers (smokers and non-smokers)

Written consents were obtained from all the volunteers prior to collection of blood. All donors used in the study were male and in the age group of 25 to 35 years. Smokers used in this study had a smoking habit for five years. Nonsmokers used as control had no prior history of smoking for the last ten years. Venous blood was taken and serum was isolated.
Exposure of guinea pigs to cigarette smoke

The procedure was essentially same as that described before. Briefly, male short hair guinea pigs weighing 350–450 g were fed ascorbate free diet for 7 days to minimize the vitamin C level of the tissues. This is because vitamin C is a potential inhibitor of CS-induced tissue damage (Panda 1999; Panda 2000; Banerjee 2008). After 7 days of vitamin C deprivation, the guinea pigs were subjected to cigarette smoke exposure from 5 Kentucky research cigarettes 3R4F (2 puffs/cigarette/animal/day) in five sessions in a smoke chamber, along with supplementation of vitamin C (1 mg/animal/day) to prevent onset of scurvy in the guinea pigs. The procedure was essentially same as that described before (Banerjee 2008). The smoke chamber used was a vacuum desiccator (2.5 litre) with an open tube at the top and a side tube fitted with a stopcock. The cigarette placed at the top was lit and CS was introduced into the chamber containing the guinea pig by applying a mild suction through the side tube for 5 sec. Thereafter, the vacuum was turned off and the guinea pig was further exposed to the accumulated smoke for another 45 sec. Pair-fed sham controls were subjected to air exposure instead of CS under similar conditions. At the end of the experimental period, the guinea pigs were euthanized using i.p. injection of ketamine hydrochloride (100 mg/kg guinea pig); right lung and blood serum was stored at -70°C. Left lung was inflation fixed overnight by 10% buffered formalin at a constant pressure of 25 cm water column.

Intramuscular injection of p-BQ to guinea pigs

As for CS exposure, animals were fed with ascorbate free diet and freshly prepared aqueous solution of p-BQ was injected intramuscularly into the lumbar muscle with 1 mg vitamin C in freshly prepared aqueous solution was given orally every day. Amounts of p-BQ used were 5 µg, 10 µg and 20 µg per day in sterile milli Q water in a final volume of 100 µl for 56 days. After the injection for specified days, the animals were sacrificed.

Calculation of p-BQ dosage

For CS exposure, two puffs per cigarette were used in a closed chamber of 2.5 litre volume. Five cigarette smoke exposures were given to each of the animals. Now, each Kentucky research cigarette 3R4F contains 100 µg of p-BSQ (Dey 2010). Respiratory minute volume of
guinea pig is approximately 160 ml per minute (Fernandes 2009). Animals were kept within the chamber for 90 seconds, 45 minutes for each exposure. So, during each exposure, smoke from half of the cigarette was present within the chamber. So the amount of p-BSQ within chamber should be 50µg per 2500 ml, or 0.02µg per ml. As the duration of exposure is 90 seconds, the animal inhales 240 ml of smoke, which will be equivalent to 4.8 µg or almost 5 µg p-BQ. For five exposures, total amount of p-BSQ inhaled will be 25 µg, which is the dose of p-BQ used for experiment is 25 µg.

Preparation of lung homogenate

A portion of the lung was weighed, homogenized in a glass homogenizer in lysis buffer containing 50 mM Tris pH-7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1% Triton X and protease inhibitor cocktail. Homogenate was centrifuged at 10,000 g at 4°C for 15 minutes and supernatant was used.

Zymography

Lung homogenate was resolved in 8% SDS PAGE with 2.5 mg/ml porcine skin gelatin in denaturing condition but at non reducing condition. After electrophoresis, the gel was washed for 30 minutes in 2.5% triton X, followed by wash in 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM CaCl₂, 0.03% NaN₃ and 2.5% Triton X for 30 minutes. The last was given using same buffer but without Triton X. The gel was incubated in fresh buffer of same composition without Triton X for 24 hours to allow gelatinolytic activity of lung homogenate to degrade gelatin matrix of the gel. Later the gel was stained with 0.05% coomassie brilliant blue in 50% methanol and 10% acetic acid and destained with methanol (50): acetic acid (10).

Incubation of HSA with aqueous extract of CS (AECS) and p-BQ

Aqueous extract of CS (AECS) was prepared as described before (Panda 1999). Briefly, smoke from one Kentucky research cigarette 3R4F was extracted with 1mL of 50 mM potassium phosphate buffer, pH 7.4, filtered through 0.22 µm Millipore filter and the pH adjusted to 7.4. The method of preparation of AECS was so devised as to simulate the manner in which the respiratory tract lining fluid is exposed to CS during the process of smoking by humans. The AECS solution thus obtained was used immediately. For experiments with AECS or p-BQ-
treated HSA, HSA (1 mg) was incubated with 50 µl of AECS or 5 µg p-BQ in a final volume of 200 µl 20 mM potassium phosphate buffer (pH 7.4).

**Immunoblot analysis**

Reaction mixtures containing 15 µg protein equivalent of HSA and lung tissue were resolved on 10% SDS-PAGE and transferred to PVDF membrane (Santa Cruz Biotechnology, inc.; sc-3723) and blocked with 5% non-fat dry milk (Himedia RM-1254). Primary antibody used was a double affinity purified polyclonal antibody raised in rabbit against a conjugate of bovine serum albumin and p-BQ (supplied by Abexome Biosciences Pvt. Ltd., India). Blots were then incubated with goat anti rabbit IgG-HRP secondary antibody (GeNei, 105499). The membranes were finally detected by chemiluminescence (LumiGLO Reagent and Peroxide, Cell Signaling Technology).

**Oxyblot analysis**

Lung homogenate (15 µg) was taken in 5% SDS and 10 µl 10 mM DNPH in trifluoroacetic acid was added to this. The reaction mixture was incubated in dark for 15 minutes in room temperature and at the end of the reaction, 10 µl neutralization solution containing 1.5 M Tris (pH 8.8) and 30% glycerol was added. β-marcaptoethanol was added to the reaction mixture and protein was resolved in SDS-PAGE. The rest procedure is same as mentioned for western blot. Primary antibody used was against carbonyl DNP derivative.

**Densitometric analysis**

Densitometric analysis of the Immunoblots was carried out by Adobe Photoshop 7.0 on reverse images.

**Histology**

Inflation fixed paraffin embedded lung histological sections were deparaffinized by xylene and rehydrated using different gradations of alcohol (100%, 95%, 90%, 70%, 50% and water). After washing thoroughly, the sections were permeabilized using permeabilization solution (100 mM citrate, 0.1% TritonX-100) and washed in water. Hematoxylin Eosin staining was done following standard procedure and sections were mounted. Slides were used for histological examination of the tissues and for morphometric analysis.
**Morphometric analysis**

Lung damage caused by exposure to CS was quantified by measuring the mean linear intercept (Lm) and destructive index (DI) as described before (Figure 1) (Dey 2010). After capturing representative fields by digital Digieye 330/210 camera and Dewinter Biozard 4.1 software, grids were laid on the fields and the number of times alveolar structure intersected the lines (Lm) were calculated. For DI calculations, alveolar abnormalities were identified just beneath the crosshairs of lines. Formula used for Lm was,

\[
Lm = \frac{L}{X \times M}
\]

Where Lm is mean linear intercept, L is total length of all lines, X is total number of intercepts in all lines and M is the magnification.

For calculation of DI,

\[
DI = \frac{D}{D+N} \times 100
\]

Where DI is destructive index; D is the destruction and N indicating normal architecture.

**Immunofluorescence of 8-hydroxy-2'-deoxyguanosine**

Following procedure described under Histology sections, after permeabilization the sections were washed with water followed by PBS wash and blocked using 10% BSA in a humid chamber for overnight at 4°C. Following day, primary antibody (ab48508) was given at a
concentration of 10 μg per ml. After incubation in a humid chamber for overnight at 4°C the sections were washed and anti mouse secondary antibody FITC conjugated were added and slides were incubated at room temperature for 45 minutes. After washing, the section nuclei were counter stained by 4', 6'-diamidino-2-phenylindole (DAPI) and mounted. Images were captured using a fluorescence microscope (Olympus Bx40) at an excitation wavelength of 488 nm for FITC and 350 nm for DAPI by cool CCD camera (Olympus; magnification, x40).

**TUNEL assay**

Paraffin embedded tissue sections (5μm) were deparaffinized, hydrated, washed and incubated in permeabilization solution (100 mM citrate, 0.1% TritonX-100). The reaction was carried out using "in situ cell death detection kit, fluorescein" (Roche) according to manufacturer's instruction. After permeabilization, the slides were washed with PBS and DNA fragmentation was detected by labeling with fluorescein labelled dUTP using terminal deoxynucleotidyl transferase. The cells were observed using a fluorescence microscope (Olympus Bx40) at an excitation wavelength of 488 nm and images were captured with cool CCD camera (Olympus; magnification, x10). The nuclei were counted by counter staining with 4', 6'-diamidino-2-phenylindole (DAPI) at excitation wavelength, 350 nm. Five fields per section of four independent sections in each group were examined.

**Identification of the mass of intact proteins by MALDI-MS**

HSA was incubated with p-BQ at different molar ratios (HSA-p-BQ ~ 1:1 to 1:60) in 20 mM ammonium bicarbonate buffer, pH 7.4, for two hours at 37°C and then dialyzed in the same buffer overnight. The dialyzed material was lyophilized. The lyophilized samples were dissolved in sample diluents (60% acetonitrile containing 0.1% TFA) to obtain a protein concentration of 1 mg/ml and 10 μl of this protein solution was desalted by reverse-phase ZipTip C18 pipette tips (Millipore, USA) and applied to the MALDI sample plate and mixed with equal volume of α-cyano-4-hydroxycinnamic acid (CHCA) matrix directly on the target plate. Samples were allowed to dry for several minutes before MALDI-TOF MS measurements were performed. Spectra of intact protein masses were obtained on an MALDI-TOF/TOF mass spectrometer (Model: 4800 MALDI-TOF-TOF, Applied Biosystems, USA). Mass spectra were recorded in the m/z range between ~20 and ~200 kD.
MALDI-TOF-TOF-MS analysis of HSA-p-BQ adduct in smokers’ serum

Coomassie (R-250 / Biosafe) stained protein bands of interest were cut manually from SDS-PAGE gel and placed into microcentrifuge tubes. Proteins were digested using In-gel tryptic digestion kit (PIERCE) and processed according to the manufacturer’s instruction. Briefly, the digested peptides were dried in vacuum and subjected to ZipTipC18 purification for MS analysis. This was followed by identification of proteins using a MALDI-TOF-TOF mass spectrometer (Model: 4800 MALDI-TOF-TOF, Applied Biosystems, USA) equipped with a special analysis software GPS. All searches were performed against suitable databases.

Spectrophotometry of p-BQ conjugates with amino acids and N-acetyl amino acids

Each of the common twenty amino acids and N-acetyl amino acids (500 nmoles) was incubated with or without 100 nmoles of p-BQ in a final reaction volume of 200 μl for two h at 37°C in 20 mM potassium phosphate buffer, pH 7.4. The N-α-acetyl amino acids used were N-α-acetyl Ala, N-α-acetyl Cys, N-α-acetyl Trp, N-α-acetyl Arg and N-α-acetyl His, N-α-acetyl Lys and N-ε-amino Lys. Before incubation, the pH of the amino acid solution of Lys, Arg, His, Asp and Glu was adjusted to 7.4. The reaction mixture obtained after incubation of the respective amino acid with p-BQ as well as the control were diluted to 1 ml by adding 800 μl of 20 mM potassium phosphate buffer, pH 7.4, and spectra were taken in the range of 200 nm to 700 nm.

Estimation of free amino groups of HSA and HSA-p-BQ adducts by fluorescamine fluorescence

After incubation of HSA with p-BQ in the molar ratio of 1:1 up to 1:60 as described above under MALDI-MS, the reaction mixture was added to 1.35 ml of sodium borate buffer (200 mM, pH 8.5) and mixed with 180 μl of fluorescamine (1mM in acetone). After incubation at room temperature for 15 min in the dark, fluorescence was measured. Taking the fluorescamine fluorescence of unreacted HSA at 474 nm as 100%, the decrease in fluorescence was measured (Colombo 2010).

In-solution digestion and identification of peptide-p-BQ complex by MALDI-TOF-TOF-MS

Ten μg of HSA p-BQ adduct in the molar ratio of 1:1 was dissolved in 15 μl of 50 mM ammonium bicarbonate (ambic) buffer and in-solution tryptic digestions (200 ng trypsin, at 37°C for overnight) were performed after the reduction/alkylation of the samples using
dithiothreitol (DTT: 100/200 mM at 95 °C for 5 min) and iodoacetamide (IAA: 200 mM at ambient temperature for 20 min in dark). The digested peptide fractions were vacuum-dried and dissolved in 10 μl of sample diluent (60% acetonitrile containing 0.1% TFA) and desalted by reverse phase ZipTip C18 pipette tips (Millipore, USA) prior to applying on to the MALDI sample plate. A small fraction (0.4 μL) of the sample was spotted on the well first and equal volume of α-cyano-4-hydroxycinnamic acid (CHCA) matrix was overlaid with a hydrophobic mask on each spots and allowed to crystallize at room temperature. MALDI-TOF-TOF measurements in the positive reflector mode were performed with an ABI 4800 Proteomics Analyzer (Applied Biosystems, USA). The laser wavelength was 355 nm, and the laser repetition rate was 200 Hz. The mass spectra were externally calibrated with the autodigest peaks of trypsin (MH+, 906.505, 1020.504, 1153.574, 2163.057 and 2273.160 Da). The MS/MS data were acquired and processed using the GPS software and MASCOT or SWISS-PROT was used to search the databases to identify the peptides. The following parameters were used in the searches: trypsin digest (one missed cleavage allowed); mass accuracy 0.2 Da, and a mass tolerance of 50 ppm was used for the peptide search; taxonomy: Homo sapiens; acetylation of the N-terminus, alkylation of cysteine by carbamidomethylation and oxidation of methionine were considered as possible modifications. MALDI-TOF-TOF measurements in the positive reflector mode were performed with an ABI 4800 Proteomics Analyzer (Applied Biosystems, USA).

**Fluorescence and absorption spectra of the HSA-p-BQ adduct**

HSA (131 μmoles) was incubated with or without different concentrations of p-BQ ranging from molar ratio of 1:1 to 1:60 in 20 mM potassium phosphate buffer (pH 7.4) at 37°C for two hours. After incubation the reaction mixture was subjected to overnight dialysis against buffer at 4°C to remove any free p-BQ or hydroquinone (HQ), a metabolite of p-BQ produced after interaction with HSA. The dialyzed reaction mixture was used for further studies. For time dependent absorbance spectrum analysis, HSA was incubated with p-BQ (1:60) and small aliquots were drawn from the reaction mixture at different interval of time to prepare a 2.6 μM solution and absorbance spectrum was acquired.
Fluorescence and absorption spectra of peptide-p-BQ complex

Each custom-made decapeptide (308 nmoles) was incubated with 1540 nmoles of p-BQ (in the molar ratio of 1:5) in 20 mM potassium phosphate buffer, pH-7.4, at 37°C in a final volume of 200 µl for two hours. Controls were incubated separately using only peptide. Then the reaction mixture was extracted successively twice with n-butanol (200 µl each time) to remove any unreacted p-BQ or hydroquinone (produced from p-BQ) present in the reaction mixture. The upper layer of butanol was discarded and the aqueous layer obtained after second butanol extraction was used for both spectrophotometry and fluorescence study.

Fluorescence studies

Unless mentioned otherwise, all the fluorescence samples were excited at a wavelength of 295 nm for selective excitation of tryptophan residues. The emission spectra were recorded from 310 to 450 nm. The path length of the cuvette used was 0.5 mm to minimize the inner filter effect. For the same reason, the protein concentration used was 0.5 µM and the optical densities of the samples were kept below 0.1.

Fluorescence Lifetime

Fluorescence lifetime measurements were determined from the total emission intensity decays of 10 µM reaction mixtures, using a nanosecond time-domain fluorimeter and operated in the time-correlated single-photon-counting mode. Excitation was provided by a pulsed, high-pressure (1.5 atm) N₂ lamp operating at 25 kHz, the FWHM of the excitation pulse profile being 1.3 ns. The N₂ emission line at 297 nm was used to excite Trp, while the emission was monitored at 340 nm. Slits of bandwidth 16 nm were used in both excitation and emission channels. Mean fluorescence lifetimes were calculated using the relation \( \langle \tau \rangle = \Sigma (a_i \, \tau_i^2) / \Sigma (a_i \, \tau_i) \), where \( a_i \) represents the fractional contribution to the time resolved decay of the component having lifetime of \( \tau_i \), the decay curve was fitted to three exponential decay with least chi-square value.

CD Analysis

Reaction mixtures prepared for the measurements of fluorescence and absorption spectra of HSA-p-BQ adducts, as described above, was diluted to 5 µM for far UV CD analysis and 10 µM for near UV CD analysis. CD spectra were recorded on a Jasco J720 spectropolarimeter by scanning reaction mixtures from 200 nm to 250 nm using 1 mm path length cuvette and 250 nm to 320 nm for near UV region using 10 mm cuvette path length. Mean residual ellipticity
(MRE) was calculated using the formula, \[ \text{MRE} = \frac{100 \cdot \text{Observed}(\theta) \cdot M_w}{[10 \cdot C \cdot n \cdot l]} \text{ in deg cm}^2 \text{ dmol}^{-1}, \]
where \( \theta \) is the observed ellipticity in millidegree, \( M_w \) is the molecular weight in KD, \( C \) is the concentration in mg/ml, \( n \) is the number of amino acids and \( l \) is the path length in cm. The \( \alpha \)-helix percentage was calculated using MRE value at 222 nm according to the formula, \( \frac{(\text{MRE}-2340)/30300} \cdot 100 \) (Chen 1972).

**ANS binding**

To 1 \( \mu \)M reaction mixture containing HSA and HSA-p-BQ complex, ANS was added from a stock of 1 mM solution to achieve a final concentration of 10 \( \mu \)M. ANS binding was studied using 420 nm as the excitation wavelength and 482 nm as emission wavelength.

**Differential scanning calorimetry (DSC) Analysis**

DSC measurements were performed using a nanocalorimeter, (N-DSC II, Calorimetry Sciences Corp, Utah U.S.A.). Pure and dialyzed samples of HSA-p-BQ adduct or native HSA (3mg/ml; 220.6 \( \mu \)M) in phosphate buffer (pH 7.4) were heated from 25\(^\circ\)C to 100\(^\circ\)C at the rate of 1\(^\circ\)C/min increment using the same buffer as blank. DSC data were corrected for instrument baselines (determined by running the dialysis buffer in both reference and sample cells just prior to placing protein in the sample cell) and normalized for scan rate and protein concentration. Data analyses were performed with Cpcalc software (Calorimetry Sciences Corp, Utah U.S.A.).

**Ligand Binding**

**Myristic Acid**

The stock solution of MA (1 mM) was prepared in 20 mM potassium phosphate buffer, pH 7.4, by heating at 50\(^\circ\)C followed by cooling to room temperature. Myristic acid was added from the stock solution in 3\( \mu \)L aliquots up to a final concentration of 51 \( \mu \)M to 1 \( \mu \)M native HSA or HSA-p-BQ adduct. The excitation wavelength was 295 nm and the emission recorded at 340 nm.

**Quercetin**

Since quercetin has substantial optical density at 295 and 340 nm that may cause inner filter effect during titration by quenching method, we employed fluorescence anisotropy method which is suitable for measuring protein-ligand dissociation constant and much less sensitive to
inner filter effect than quenching. A 10 mM concentrated stock solution of quercetin was prepared in ethanol. From this stock solution, further diluted stock solution of quercetin was prepared in buffer. In a final volume of 500 µl, 5 µM quercetin was incubated with increasing concentrations of protein up to 50 µM. Amount of ethanol in the final incubation system was less than 1%.

**Paracetamol**

A 50 mM stock solution of pure paracetamol was prepared by dissolving paracetamol in buffer. The concentration was determined using 10236 molar absorptivity of paracetamol at 243 nm (Abdellatef 2007). Small aliquots were added from diluted paracetamol stock to a 0.5 µM free HSA or HSA-p-BQ complex up to a concentration of 1.9 mM. The Tryptophan fluorescence quenching was measured using an excitation wavelength of 295 nm and measuring emission at 340 nm.

**Determination of dissociation constant**

**Anisotropy measurements**

Steady-state anisotropy was recorded with a Hitachi model F-7000 spectrofluorometer equipped with a polarization accessory. Excitation of quercetin was done at 400 nm. The fluorescence anisotropy (A) values were obtained using the expression $A = (I_{vv} - 2I_{vh})/(I_{vv} + 2I_{vh})$, where $I_{vv}$ and $I_{vh}$ are the vertically and horizontally polarized components of probe emission at 550 nm with excitation by vertically polarized light at 400 nm and G is the sensitivity factor of the detection system. The excitation and emission slits were 5 and 10 nm, respectively. The quercetin concentration was 5 µM and HSA or HSA-p-BQ concentrations were varied between 1 (Bartecchi 1994) to 50 µM (Sengupta 2002). The data were fitted according to the following equation (Heyduk 1990):

$$A = A_L + (A_{PL} - A_L) \times \left[ \left( L_T + P_T + K_d \right) - \left( \left( L_T + P_T + K_d \right)^2 - 4L_T P_T \right) \right]^{1/2} / 2L_T P_T$$

where A is the measured value of anisotropy; $A_L$ and $A_{PL}$ are specific values of anisotropy associated with free quercetin and HSA-quercetin or HSA-p-BQ-quercetin complex, respectively. The $K_d$ value was obtained by fitting the experimental data to the equation by using
least-squares method using KYPLOT (Koichi Yoshioka, 1997–2000, version 2.0, beta 13) with \( A_L, A_{PL} \) and \( K_d \) as floating parameters.

**Fluorescence quenching method**

The values of the dissociation constant (\( K_d \)) for the HSA-ligand complex (P.L) was determined by assuming a simple bimolecular binding equilibrium between the HSA (P) and ligand, L, using the nonlinear least-squares method according to the following equation (Takita 1996).

\[
\Delta F(\%) = \Delta F_{\text{max}} \frac{[L]}{K_d + [L]_0}
\]

Where \([L]_0\) is the total concentration of the ligand, \([L]_0 = [L] + [P.L]\), and \(\Delta F\) is the fluorescence intensity change observed at 340 nm when a certain amount of the ligand is added. This is expressed as a percentage of the fluorescence intensity of the HSA; namely \(\Delta F(\%) = 100 \times \frac{F_{PL} - F_0}{F_0}\), where \(F_{PL}\) and \(F_0\) are the fluorescence intensities at 340 nm of the HSA-ligand complex and the HSA, respectively. The data were fitted using KYPLOT (Koichi Yoshioka, 1997–2000, version 2.0, beta 13) with \(\Delta F_{\text{max}}\) and \(K_d\) as floating parameters.

**Statistical analysis**

All the data were analyzed by one way ANOVA test. The data (Mean, SD and N, the number of observations) were entered in the appropriate boxes in the one way ANOVA calculation webpage http://statpages.org/anovasm.html. The p values were calculated using appropriate F tests. Difference with p values <0.05 was considered significant.