Materials & Methods
MATERIALS AND METHODS

Sperm Samples

Human semen samples were collected from healthy donors through the courtesy of the General Hospital, Sector 16, Chandigarh. Sperm concentration, motility and forward progression were evaluated subjectively at room temperature as percent motile cells using an improved Neubauer's haemocytometer. Samples having a final concentration of $80 \times 10^6$ sperm cells/ml and with more than 70% motile cells were selected. Seminal plasma was discarded by centrifuging the samples at 300 xg. The pellet so obtained was suspended in an equal volume of phosphate buffered saline (PBS) containing 0.9% sodium chloride, 0.2M monobasic sodium phosphate and 0.2M dibasic sodium phosphate, pH 7.2 (see appendix). This suspension was used throughout the investigation.

Drug Preparation

Nicotine (Mol. Wt.: 162.0) and caffeine (Mol. Wt.: 194.2) were purchased from Sigma chemical Co., St. Louis, MO, USA. The stock solutions of nicotine and caffeine were prepared by dissolving in 0.2 M PBS (pH 7.2) to a concentration of 100mM. Final concentrations of 0.5 and 1 mM in case of nicotine and 5, 7 and 9 mM in case of caffeine were prepared by diluting the stock solution suitably with 0.2 M PBS (pH 7.2) and were used in various experiments.

Preparation of Post Mitochondrial Supernatant

The spermatozoal samples suspended in 0.2 M PBS (pH 7.2), were gradually cooled at 4°C and homogenized in an ice bath using Potter-Elvehjem homogenizer. The homogenate was centrifuged first at 1,000 rpm and finally at 10,000 rpm for 10 min each at 4°C. The supernatant was filtered through a plug of glass wool to remove the floating lipids. The resultant solution was treated as post mitochondrial supernatant (PMS) in the respective experiments.
Preparation of Antioxidant Solutions

**Ascorbic Acid (AA)**

The vitamin was purchased from Loba chemie, PB No. 2042, Mumbai, India. Stock solution of ascorbic acid (Mol. Wt.: 176.13) was prepared by dissolving it in PBS (pH 7.2) to the concentration of 20 mM. Various final concentrations ranging from 0.6 to 2 mM were prepared and used in the experiment.

**Glutathione (GSH)**

Glutathione was purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India. Reduced glutathione (GSH) (Mol. Wt.: 307.33) stock solution was prepared by dissolving it in PBS (pH 7.2) to the concentration of 100 mM. Final concentrations of 2 to 10 mM were used.

**Trolox (a water-soluble analogue of α-tocopherol)**

Trolox was purchased from Aldrich Chemical Co., Milwaukee, USA. The stock solution was prepared by dissolving trolox (Mol. Wt.: 250.29) in PBS (pH 7.2) to the concentration of 5 mM. Final concentrations ranging from 10 to 30 μM were used.

**Sources of other chemicals**

Unless otherwise noted, analytical reagent grade chemicals were used in all the experiments without further purification. The standards used in each of the analyses were specified by the manufacturers as being chromatographically homogenous. The common chemicals such as Tris, NaCl, KCl, PCA, TCA, FeCl₃, ANSA, EGTA, MgCl₂, NaH₂PO₄, Na₂HPO₄, fructose, sodium citrate, FeSO₄, Copper tartrate, Na₂CO₃, etc. were obtained commercially from the reputed Indian companies. Certain chemicals were purchased from the following sources: ATP, GSH, TBA, CDNB, DTNB, DMSO, Ethidium bromide, Triton-X-100: SRL Company, SISCO research laboratories, Pvt. Ltd., Bombay, India; NADP, NADPH, Glutaraldehyde, Ouabain, Glucose-6-phosphate: Fluka, Biochemka AG CH-9470, Buchs, Switzerland; GSSG, Cysteine-Glycine, L-glutamate, L-α-amino-butyrate: Sigma, Chemical Company, St. Louis, MO, USA.
Materials & Methods

Measurement of Lipid Peroxidation

Method adopted: Buege and Steven (1978)

Reagents

150 mM Tris-HCl buffer (pH 7.2) : 1.817 g of Tris-HCl dissolved in 100 ml of d.w. The pH was adjusted at 7.2.

10% TCA : 10 g of TCA dissolved in 100 ml d.w.

2% TBA in 0.05 N NaOH : 200 mg NaOH dissolved in 100 ml of d.w. and added 2 g of thiobarbituric acid (TBA) to this solution.

Procedure

Thiobarbituric acid (TBA) reactivity was used as an index of lipid peroxidation (LPO) in the control and test series by reacting malonyldialdehyde (MDA) and TBA to form MDA-TBA complex. 1 ml of assay mixture in untreated-unsupplemented series containing Tris-HCl buffer (150 mM), 0.1 ml of sperm sample and adequate amounts of PBS (pH 7.2) was incubated at 37°C for 30 minutes in a water bath. In the drug-treated samples, 0.1 ml of 0.5 and 1 mM solutions of nicotine and/or 5,7, and 9 mM solutions of caffeine was added. 0.1 ml of antioxidants alone and/or in combination also was added to the assay mixture of untreated and treated series in another set of experiments as supplementation while an equal volume of PBS (pH 7.2) was added to the untreated-unsupplemented series. The reaction was stopped by adding 1 ml of chilled 10% TCA. Samples were then centrifuged at 2500 xg for 10 minutes and an aliquot of 1 ml was taken out into another set of tubes containing 1 ml of 2% TBA in 0.05 N NaOH. The tubes were vortexed thoroughly and immersed in boiling water bath for 10 minutes and cooled at room temperature. The absorbance of the coloured solution was measured at 532 nm. Since, malonyldialdehyde may not be the only species detected in this assay, the data represent the absorbance changes observed at 532 nm, that is the absorption maxima of the adducts produced. Finally, the rate of LPO was calculated as nmol MDA. mg prot⁻¹. min⁻¹.
Experiments were performed using:

1. Different concentrations of ascorbic acid (0.6 to 2 mM), glutathione (2 to 10 mM) and trolox (10 to 30 µM) in untreated samples to record their antioxidant abilities against formation of MDA-TBA complex.

2. Selected concentrations of above mentioned antioxidants (single and/or in combination) in untreated as well as in nicotine (0.5 and 1 mM) and caffeine (5, 7 and 9 mM) treated series.

3. Antioxidants (single and/or in combination) in drug-treated and untreated samples supplemented with 0.1 ml of 0.2 mM ferrous sulphate (FeSO₄).

4. Different concentrations of FeSO₄ (25-200 µM) and ascorbic acid (125-1000 µM), all in ratio of 1:5, as LPO inducing system for substrate kinetic analysis.

**Estimation of Proteins**

The extent of MDA production and the activities of enzymes were calculated and expressed per mg protein. The amount of protein in the sample was estimated by the sodium dodecyl sulphate-Lowry method of Lees and Paxman (1972) using BSA (Cohn Fraction V) as standard.

**Principle**

In this method the cell membranes are disrupted to release the bound proteins using a detergent, in this case SDS. The deep blue colour developed at the end is due to two reactions: (i) the coordination of peptide nitrogen atoms with copper II ions and (ii) the reduction of Folin-Phenol reagent which contains phosphomolybdate and phosphotungstate, by tyrosine and tryptophan residues in the proteins.

**Reagents**

5% SDS in 0.5 N NaOH : Appendix.

Copper carbonate solution : Appendix.
Folin-Phenol reagent : 2 N Folin-Phenol reagent diluted 1:1 with d.w.

Standard BSA (200µg/ml) : 2 mg of BSA dissolved in 10 ml of d.w.

**Procedure**

0.9 ml of 5% SDS in 0.5 N NaOH was added to 0.1 ml of sperm sample in test tubes. The tubes were allowed to remain at room temperature for atleast 3 hours and were agitated 2-3 times in a Vortex mixer to make sure that the samples were dissolved thoroughly. To this, 2.5 ml of copper carbonate solution was added and tubes were allowed to stand for 15-20 minutes. Then, 0.25 ml of Folin-Phenol reagent was added, samples were mixed immediately and allowed to stand for 45 minutes. The intensity of blue colour developed was read at 750 nm. BSA standards (20-200µg/ml) was also run simultaneously.

**Analysis of Spermatozoal Membrane Lipids**

*Extraction of membrane lipids*

A total volume of 2 ml assay mixture in different series contained:

a) 1.4 ml spermatozoal membrane suspension + 0.6 ml of 0.2M PBS (control).

b) 1.4 ml spermatozoal membrane suspension + 0.6 ml of drugs and 0.2M PBS.

c) 1.4 ml spermatozoal membrane suspension + 0.6 ml of antioxidants (single/in combination) and 0.2 M PBS.

d) 1.4 ml spermatozoal membrane suspension + 0.6 ml of drugs plus antioxidants and 0.2 M PBS.

each series was incubated at 37°C for 30 minutes and then for each the following procedures were carried out to extract the respective lipid contents.

*Method adopted* : Folch et al. (1957)
**Procedure**

The resulting assay mixture (2ml) was mixed thoroughly in a flask with 20 volume of chloroform: methanol (2:1, v/v) and the mixture left for 15 minutes at 45°C. The mixtures were then taken out and cooled at room temperature. 4 ml of chilled chloroform: methanol (2:1, v/v) added. The content was thoroughly agitated and ground in a pestle and mortar set, and then filtered through a Whatman No. 1 filter paper into a graduated cylinder. The residue left on the filter paper was washed three times with 10 ml of chloroform: methanol (2:1, v/v). 0.9% KCl was added (20% of the total volume of extract) to the extract. The contents were mixed vigorously and allowed to stand overnight in cold so as to separate the aqueous and lipid layers clearly. Upper aqueous phase was removed by aspiration with a Pasteur pipette and the lower layer was washed three times with 2 ml of chloroform: methanol: 0.9% KCl, 3:48:47, v/v. The washed lower layer was transferred to a round bottom flask and evaporated to dryness under vacuum at a temperature below 45°C while the upper aqueous layer was added each time to the previously separated upper phase to estimate the lipid bound sialic acid fraction. To the residue, 2 ml of chloroform: methanol: water, 64:32:4, v/v, was added and evaporated again to dryness. The process was repeated two times. Dried lipids were redissolved in chloroform and filtered once again. The filtrate was dried in a rotary vacuum evaporator (Buchi type, Metrex, New Delhi) under reduced pressure and at a temperature of not more than 45°C. A known volume of chloroform: methanol (2:1, v/v) was added to redissolve the lipids in a tightly closed container and used as such for various lipid estimations. The lipid extract may be stored at -20°C to avoid any change in the volume of the solvent and also prevent oxidative changes but it was usually analysed within a fortnight.

**Estimation of total lipids**

*Method adopted:* Fringes and Dunn (1970)
**Principle**

The colorimetric reaction for lipid estimation requires a double bond. H$_2$SO$_4$ reacts with -C=C- and gives a carbonium ion which forms a coloured complex with a phosphate ester of vanillin (colouring reagent).

**Reagents**

- Phosphovanillin reagent: 80 ml of orthophosphoric acid + 20 ml of 0.6% vanillin (made fresh).
- Olive oil standard: 5 mg/ml in absolute ethanol.

**Procedure**

To 0.1 ml of the lipid extract, 2 ml of conc. H$_2$SO$_4$ was added in carefully washed test tubes (washed scrupulously in chromic acid but avoiding detergents). Tubes were shaken vigorously and kept in a boiling water bath for 15 min, which were then cooled at room temperature. 5 ml of phosphovanillin reagent were added in different sets of tubes to which 0.2 ml of the contents of the cooled test tubes were added, mixed properly and allowed to stand at room temperature for 30 min. The optical density (OD) of the coloured complex was read at 510 nm. Blank and standard (in the form of olive oil 500-5000 µg/ml) were also put simultaneously.

**Estimation of total phospholipids**

Total phospholipids were obtained by estimating inorganic phosphorus as obtained after digesting the phospholipids and multiplying it by a factor of 25 assuming an average molecular weight of the phospholipids to be 775. The figure of 25 was derived by dividing 775 by 31, the molecular weight of phosphorus.

**Estimation of phosphorus**

*Method adopted:* Bartlett (1959), modified by Marinetti (1962)
Principle

When acid hydrolysate of any substance containing phosphorus is treated with molybdate, it forms phosphomolybdic acid, which in turn is reduced by the addition of 1,2,4 aminonapthol sulphonic acid (ANSA) to produce a blue colour whose intensity is directly proportional to the amount of phosphorus.

Reagents

Phosphorus standard : Stock sol.: 1 mg Pi/ml (439.4 mg KH$_2$PO$_4$/100 ml d.w.). Working sol.: 10 μg/ml.

ANSA reagent : Appendix.

2.5% acid ammonium molybdate : Appendix.

Procedure

Various concentrations of standard KH$_2$PO$_4$ (1-5 μg Pi/ml) and 0.1 ml lipid extract were taken in different test tubes. 0.9 ml of perchloric acid (PCA) was added and the tubes were kept in hot sand bath and digested for 10-15 min till the solution became colourless. Hard glass test tubes were used for this purpose to avoid cracking at a very high temperature. The test tubes were covered with aluminium foil so that the amount of PCA fumes which escape was kept to minimum. The solution was then cooled at room temperature and 7 ml of double distilled water were added. 0.5 ml of 2.5% ammonium molybdate and 0.2 ml of ANSA were added sequentially with thorough mixing of the contents. The mouth of the tubes was covered with aluminium foil and the tubes were kept in boiling water bath exactly for 7 min. After cooling, the OD of the blue colour thus obtained was measured at 830 nm. Tubes were always scrupulously washed, rinsed in d.w. and other precautions were taken to avoid any contamination of phosphorus from sources other than the samples, which otherwise gives a very high reading as detected from the blank tubes.
Materials & Methods

Estimation of Cholesterol

Method adopted: Zlatkis et al. (1953)

Principle
In the presence of conc. \( \text{H}_2\text{SO}_4 \) and glacial acetic acid, cholesterol forms a coloured complex with \( \text{FeCl}_3 \) that can be measured spectrophotometrically at 540 nm.

Reagents
Ferric chloride reagent: Stock sol.: 10% anhydrous \( \text{FeCl}_3 \) in glacial acetic acid. Working sol.: 1 ml of stock sol. diluted to 100 ml with conc. \( \text{H}_2\text{SO}_4 \) (made afresh).

Cholesterol standard: 1 mg/ml of chloroform. (doubly recrystallized)

Procedure
Different concentrations of cholesterol standard (20-100 μg/ml) and 0.1 ml of the lipid extract were dried in separate tubes at 37°C. 3 ml of glacial acetic acid were added to them followed by 2 ml of the working ferric chloride reagent. The tubes were vortexed and kept in dark for 30 min. Optical density was read at 540 nm.

Estimation of free fatty acids

Method adopted: Lowry and Tinsley (1976)

Reagents
Palmitic acid standard: 0.2 mg/ml of chloroform.

Cupric acetate-pyridine reagent:
5% aqueous cupric acetate sol. was filtered and its pH was adjusted to 6.0-6.2 with pyridine.

Benzene: As it is.

43
**Procedure**

The chloroform in 0.1 ml of test solution and standard palmitic acid (25-150 µg/ml of chloroform) were evaporated to dryness. 5 ml of benzene was added and the mixture was warmed slightly. 1 ml of cupric acetate-pyridine reagent (pH 6.2) was added, mixed for 2 min and centrifuged for 5 min at low speed (600 xg). The absorbance of the upper layer was recorded at 715 nm.

**Separation of lipid fractions by thin layer chromatography (TLC)**

**Preparation of TLC plates**

Glass plates, 20x20 cm were washed, dried and rinsed with acetone prior to use. The dried plates were coated by an applicator with 0.25 mm thickness of silica gel G (Mangold, 1961). Silica gel G slurry was made by quickly mixing 30g of silica gel G and 60 ml of distilled water. The gel layered plates were allowed to dry at room temperature and were activated in an oven for one hour at 110°C before use.

**Separation of phospholipids**

25 µl of lipid extract containing about 10 µg of lipid phosphorus was spotted with a Hamilton syringe on the plates coated with silica gel G for one dimensional thin layer chromatography. The plates were kept in a rectangular jar (10”x 5”) containing solvent system consisting of chloroform: methanol : 14 N ammonia, 65:25:4, v/v as described by Sanyal (1986). Filter paper soaked with the solvent mixture was kept on the inner wall of the jar for providing proper saturation with the solvent vapours to facilitate uniform separation and also to avoid tailing. Authentic phospholipid standards were also run simultaneously.

**Detection of spots (Stahl, 1969)**

The developed plates were exposed to iodine vapours. Phospholipid fractions appeared as yellowish-brown spots after exposure to iodine vapours which were identified by comparison with the co-chromatographed standards. The spots were marked with sharp needle and the I₂ was allowed to evaporate in air.
Quantitation of phospholipids from the chromatoplates

The detected and marked spots were directly scrapped off cautiously into separate test tubes. The phosphorus content was estimated after digestion as described earlier. Silica gel G did not interfere in the development of the colour, as evident in the blanks which contained equal areas of the silica gel G but no lipid spot. Before reading the OD, the tubes were centrifuged at 1,000 xg for 10 min and the OD of the supernatant was measured at 830 nm. Phospholipid content of each fraction was calculated as described earlier.

Separation of neutral lipids

Neutral lipids were separated by TLC using the solvent system of petroleum ether: solvent ether: glacial acetic acid, 90:10:1, v/v as described by Sanyal (1986). The spots were marked after exposure to iodine vapours, scrapped off and eluted with chloroform-methanol (2:1, v/v) three times. They were centrifuged and decanted into fresh tubes, and pooled. Triglycerides, cholesterol, cholesterol ester and free fatty acids could then be estimated in the dried lipid extract according to the procedures described earlier.

Determination of Thiols

Method adopted: Ellman (1959) as described by Sedlak & Lindsay (1968)

Principle

5, 5'-dithio bis- (2-nitrobenzoic acid) (DTNB) is reduced by –SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of –SH. The nitromercaptobenzoic acid anion, an yellow colour is used to measure the –SH content.

\[
\text{R} - \text{SH} + \text{S-N=NO}_2 \rightarrow \text{R-S-S-N=NO}_2 + \text{HS-NO}_2 \rightarrow \text{R-S-S-N=NO}_2 \rightarrow \text{COOH}
\]

The amount of thiols was expressed as μ mol – SH.mg prot \(^{-1}\). min \(^{-1}\).
Materials & Methods

1. Total thiols (T-SH)

Reagents

- 0.01 M DTNB: 79.2 mg of DTNB in 20 ml of absolute methanol.
- 0.2 M EDTA: 1.65 g of EDTA in 10 ml of d.w.
- 0.2 M Tris-HCl buffer (pH 8.2) with EDTA:
  - 24.2 g of Tris in 1000 ml of d.w. +
  - 100 ml of 0.2 M EDTA, pH adjusted by 1 N HCl.

Procedure

The assay mixture containing 1.5 ml of Tris-HCl buffer with EDTA (pH 8.2), 0.5 ml of sperm homogenate, 0.1 ml of 0.01M DTNB in methanol was treated in the presence of nicotine (0.5 and 1 mM) and/or caffeine (5, 7 and 9 mM) with antioxidant supplementation. The total volume of assay mixture was raised to 10 ml with absolute methanol and the content were mixed thoroughly. The capped tubes were allowed to stand at room temperature with occasional shaking for 30 minutes. The tubes were then centrifuged at 2,500 xg for 15 minutes. The absorbance of the clear aliquot (after filtration through Whatman No.1 filter paper) was measured at 412 nm. The molar extinction coefficient (ε) at 412 nm was taken as 13,100 M⁻¹·cm⁻¹, and used in the calculation of T-SH content. Control and blank tubes were run simultaneously.

2. Non-Protein sulfhydryl Groups (NP-SH)

Reagents

- 0.01 m DTNB: 79.2 mg of DTNB in 20 ml of absolute methanol.
- 0.2 M EDTA: 1.65 g EDTA in 10 ml of d.w.
- 0.4 M Tris buffer (pH 8.2) with EDTA:
  - 48.4 g of Tris in 1000 ml of d.w. +
  - 100 ml of 0.2 M EDTA solution.
- 20% TCA (chilled): 20 g of TCA dissolved in 100 ml of d.w.
Procedure

2 ml of assay mixture contained 0.5 ml spermatozoal suspension, 1.2 ml of 20% TCA, treated with nicotine (0.5 and 1 mM) and/or caffeine (5-9 mM) and with antioxidant supplementation, each in 0.1 ml. Suitable control and blank were run simultaneously. The tubes were then vortexed and allowed to stand in cold condition with occasional shaking for 15 minutes. The samples were centrifuged at 2,500 xg for 15 minutes. 1 ml of aliquot was mixed with 2 ml of 0.4 M Tris buffer (pH 8.2) and 0.1 ml of 0.01 M DTNB. The contents were mixed well and the absorbance was read within 5 minutes of the addition of DTNB at 412 nm against a reagent blank. The molar extinction coefficient (ε) at 412 nm used, was 13,100 M⁻¹ cm⁻¹, and used for calculation as earlier.

3. Protein Bound Sulfhydryl Groups (PB-SH)

The protein bound sulfhydryl group (PB-SH) content was calculated by subtracting the non-protein bound sulfhydryl groups (NP-SH) from total thiols (T-SH).

Glutathione S-transferase (GST) (EC 2.5.1.18)

Method adopted: Habig et al. (1974)

Principle

1-Chloro-2,4-dinitrobenzene (CDNB) was used as electrophile to measure GST activity in the variously treated spermatozoal samples.

Reagents

0.2 M phosphate buffer (pH 7.0) : Appendix.

20 mM reduced glutathione (GSH) : 614.6 mg in 100 ml of d.w.

20 mM CDNB in 95% ethanol : 405.2 mg CDNB in 100 ml of 95% ethanol.

Procedure

0.1 ml of post-mitochondrial supernatant (PMS) was treated in the presence of nicotine (0.5 and 1 mM) and/or caffeine (5-9 mM). 0.1 ml of
Materials & Methods

Antioxidants (singly/in combination) was supplemented to these samples. Suitable control and blank were also run simultaneously. The pH of the assay mixture was maintained at 6.6 with the help of 0.2 M phosphate buffer. 0.1 ml of 20mM CDNB solution was added and the assay mixture was incubated at 37°C for 30 minutes. To initiate the reaction 0.1 ml of 20mM GSH was added. The reaction was allowed to proceed for 5 minutes in a cuvette at 340 nm. The increase in absorbance was recorded after every minute. The average absorption per minute (ΔAA) was calculated thereafter. The reagent blank was also run simultaneously. The activity of GST was expressed as unit amount of CDNB-GSH conjugates, mg prot⁻¹, min⁻¹. The molar extinction coefficient (ε) of GSH at 340 nm was taken as 9600 M⁻¹.cm⁻¹. Substrate kinetic studies were carried out to elucidate the nature of the effect of drug and antioxidant supplementation on GST activity using different substrate concentrations ranging from 10 to 40 mM of GSH.

Glutathione Reductase (NADPH-dependent oxidized glutathione reductase) (EC 1.6.4.2)

Method adopted: Horn (1971)

Principle

Glutathione reductase (GRD) catalyses the reduction of oxidized glutathione (GSSG) by NADPH to reduced glutathione (GSH). The high ratio (100:1) of GSH to GSSG found intracellularly is maintained by the activity of GRD.

\[
\text{NADPH} + \text{GSSG} \xrightarrow{\text{GRD}} \text{NADP}^+ + 2\text{GSH}
\]

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M phosphate buffer (pH 6.6)</td>
<td>Appendix.</td>
</tr>
<tr>
<td>9 mM EDTA</td>
<td>7.44 g dissolved in 100 ml of d.w.</td>
</tr>
<tr>
<td>30 mM GSSG</td>
<td>1.838 g dissolved in 100 ml of d.w.</td>
</tr>
<tr>
<td>0.3 mM NADPH</td>
<td>22.962 mg dissolved in 100 ml of d.w.</td>
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</tbody>
</table>
**Materials & Methods**

**Procedure**

0.1 ml of post-mitochondrial supernatant (PMS) was treated in the presence of nicotine (0.5 and 1mM) and/or caffeine (5-9 mM). 0.1 ml of antioxidants singly and/or in combination form was supplemented to these samples. Suitable control and blank were also run simultaneously. The pH of the reaction was maintained at 6.6 with 0.2 M phosphate buffer. To this, 9mM EDTA was added. The assay mixture was mixed well and incubated at 37°C for 30 minutes. The reaction was initiated by adding 0.1 ml of 30 mM GSSG and 0.1 ml of 0.3 mM NADPH solutions to the assay mixture in the cuvette. The change in optical density (ΔA) was recorded after each minute for a period of 5 minutes at 339 nm. The suitable blank (lacking GSSG) was run alongwith. For calculations, the molar extinction coefficient (ε) of NADPH at 339 nm was taken as 6.22 x 10³ M⁻¹.cm⁻¹. The results expressed as unit amount of μmol NADPH-oxidized. mg prot⁻¹.min⁻¹.

**Dipeptidase (EC 3.4.3.5)**

**Method adopted**: Hughey *et al.* (1978)

**Principle**

Cysteinylglycine $\xrightarrow{\text{Dipeptidase}}$ cysteine + glycine.

**Reagents**

- 25 mM Cysteine-glycine : 0.445 g of cysteine-glycine in 100ml of d.w.
- 2mM MnCl₂ : 9.885 mg of MnCl₂ in 100ml of d.w.
- 250 mM dipotassium phosphate (K₂HPO₄) (pH 7.0) : 1.062g of K₂HPO₄ in 100ml of d.w.
- 0.2% ninhydrin : 0.2g of ninhydrin in 100 ml of ethanol.
- 5 mM glycine (standard) : 40.05 mg of glycine (glycine) in 100 ml of d.w.

**Procedure**

The 150 μl of assay mixture contained 5mM cysteine-glycine, 0.4 mM MnCl₂, 50mM dipotassium phosphate (pH 7.0), 30μl of spermatozoal
suspension, treated with nicotine and caffeine and with antioxidant supplementation. The untreated-unsupplemented series received an equal amount of 0.2M PBS (pH 7.2). After addition of spermatozoal suspension the mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 20ml of chilled 1 N HCl. Four 8 ml aliquots were spotted on Whatman No. 1 chromatographic paper and developed in a phenol : water (75:25 w/w) solvent system adjusted to pH 7.0 with ammonium hydroxide. After drying (at 100°C in oven), the chromatographs were sprayed by 0.2% ninhydrin in ethanol and dried again at 100°C in an oven until colour (brown to violet) was developed. Appropriate areas were marked and cut out, then eluted with 5ml of 50% ethanol in a 37°C shaker water bath for 1hr. Absorbance of the elute at 570 nm was compared to a standard curve prepared from similarly processed glycine standards (1-5mM). The results were expressed as mM gly. mg prot⁻¹. min⁻¹.

γ-Glutamyl Transpeptidase (GTT) (EC 2.3.2.2)

Method adopted : Orlowski and Meister (1965)

Principle

The hydrolysis of L-γ-glutamyl-4-nitroanilide at pH 7.8 with glycylglycine as acceptor yields coloured 4-nitroaniline.

\[
\text{L-γ-glutamyl-4-nitroanilide} \xrightarrow{\text{GTT}} \text{4-nitroaniline} + \text{γ-glutamyl glycylglycine}
\]

Reagents

Buffer : Appendix.
0.2 mM PBS (pH 7.2) : Appendix.

Substrate stock solution (1mM) : Dissolved 2.672 mg of substrate in 1 ml of conc. HCl. To it 9 ml of d.w. was added.
(L-γ-glutamyl-4-nitroanilide)

Substrate working solution (100 μM) : 1 ml of stock solution in 9 ml of d.w.
Procedure

1 ml of assay mixture contained 0.6 ml of buffer (pH 7.8), 0.1 ml of spermatozoal suspension treated with nicotine and/or caffeine, with antioxidant supplementation. In case of untreated-unsupplemented or control series an equal volume of 0.2 M PBS (pH 7.2) was added. The contents were mixed well and kept at 37°C for 30 minutes. To initiate the reaction, 0.1 ml of L-glutamic acid-γ-nitroanilide (substrate working solution) was added. The contents were then mixed. The formation of p-nitroaniline was determined readily from the increase in absorbance at 405 nm after 1 minute. The absorbance was recorded for 5 consecutive minutes. A suitable reagent blank was run simultaneously. The molar extinction coefficient of p-nitroaniline at 410 nm was taken as 9900 M⁻¹.cm⁻¹. This value was employed in calculating the enzyme specific activity as follows:

\[
\text{Enzyme specific activity} = \frac{\Delta A_{405}}{\text{Min.}} \times \frac{1000}{0.1} \times \frac{1.0}{9.9} \times \frac{1}{\text{Protein content}}
\]

where, 1.0 is the final volume of assay mixture, 0.1 (ml) is the volume of spermatozoal suspension, and Min. is the time between substrate addition and first reading (1 minute). Finally, the results were expressed as μ moles p-nitroaniline. mg prot⁻¹.min⁻¹.

γ-Glutamyl Cysteine Synthetase (GGCS) (EC 6.3.2.2)

Method adopted: Sekura and Meister (1977)

Principle

\[
\text{L-glutamate + L-α-aminobutyrate + ATP} \xrightarrow{\text{Mg}^{2+}} \text{L-γ-glutamyl-L-γ-aminobutyrate + ADP + P}_i
\]

The inorganic phosphorus (Pᵢ) liberated in the supernatant was estimated during the experiment.
**Reagents**

200 mM MgCl$_2$ : 0.953 g MgCl$_2$ dissolved in 50 ml of d.w.

100 mM L-glutamate (monosodium salt) : 0.845 g in 50 ml of d.w.

100 mM L-α-amino-butyrate : 0.510 g in 50 ml of d.w.

50 mM Na$_2$-ATP : 0.146 g in 50 ml of d.w.

20 mM Na$_2$-EDTA : 0.292 g Na$_2$-EDTA in 50 ml of d.w.

1000 mM Tris-HCl (pH 8.2) : 6.060 g Tris-HCl in 50 ml of d.w.

100 μg Bovine serum albumin (BSA) : 1 mg of BSA dissolved in 10 ml of d.w.

10% TCA : 10 g of TCA in 100 ml of d.w.

**Procedure**

γ-glutamyl cysteine synthetase (GGCS) was determined in the assay mixture contained (in a final volume of 1 ml) sodium L-glutamate (10mM, final concentration), L-α-amino-butyrate (10mM), MgCl$_2$ (20mM), Na$_2$-ATP (50 mM), Na$_2$-EDTA (2 mM), Tris-HCl (100mM), BSA (10μg) treated with nicotine (0.5 and 1 mM) and/or caffeine (5,7 and 9 mM) and supplemented with antioxidants, singly/in combination. The untreated-unsupplemented samples received an equal volume of the vehicle of the drug. The reaction was initiated by adding 0.1 ml of spermatozoal suspension to the above mentioned items. After incubation at 37°C for 30 minutes, the reaction was terminated by adding 1 ml of chilled 10% TCA. The inorganic phosphorus (P$_i$) formed was determined following the method of Fiske and Subbarow (1925). The GGCS activity was expressed as unit amount of μg Pi. mg prot$^{-1}$. min$^{-1}$.

**Glutathione Peroxidase (GPx) (EC 1.11.1.9)**

**Method adopted:** Flohe and Gunzler (1984)

**Principle**

\[ 2\text{H}_2\text{O}_2 + 2\text{GSH} \xrightarrow{\text{GPx}} 2\text{H}_2\text{O} + \text{O}_2 + \text{GSSG} \]
Reagents

50 mM Potassium phosphate buffer (PPB) (pH 7.4) :
Stock Sol. A: 500 mM KH₂PO₄ — 6.8 g in 100 ml of d.w.
Stock Sol. B: 500 mM K₂HPO₄ — 8.7 g in 100 ml of d.w.

The desired pH was obtained by combining 19.80 ml of Sol. A and 80.20 ml of Sol. B. The total volume raised to 1000 ml with d.w.

0.5 mM EDTA : 14.6 mg in 100 ml of d.w.
10 mM Sodium azide : 85 mg of NaN₃ in 100 ml of d.w.
Glutathione reductase (GRD) : 0.24 I.U.
0.15 mM NADPH : 1.10 mg in 10 ml of d.w.
1 mM GSH : 3.1 mg of GSH in 10 ml of d.w.
0.15 mM H₂O₂ : 0.51 mg in 100 ml of d.w.

Procedure

GPx catalyzes the formation of GSSG from GSH and H₂O₂. The GSSG is continuously reduced by excess of glutathione reductase and simultaneous oxidation of NADPH is measured at 339 nm. One ml of assay mixture contained 50 mM PPB (pH 7.4), 0.5 mM EDTA, 10 mM NaN₃ (to inhibit the activity of catalase in media), 0.24 I.U. of GRD, 0.15 mM NADPH, 1 mM GSH, 0.15 mM H₂O₂, along with caffeine and nicotine treatments, and with antioxidant supplementation. Finally, 0.1 ml of spermatozoal suspension (PMS) was added and the mixture was well mixed and incubated at 37°C for 30 min. The change in optical density (ΔAA) was recorded every one minute at 339 nm for a period of 5 minutes. The extinction coefficient (ε) of NADPH at 339 nm was taken as 6.22 mM⁻¹ cm⁻¹ for the calculation of the enzyme activity. The results were expressed as unit amount of μ mol NADPH oxidized .mg prot⁻¹.min⁻¹.

Estimation of Inorganic phosphorus

Method adopted: Fiske and Subbarow (1925)
Principle

When acid hydrolysate of any substrate containing phosphorus is treated with molybdate, it forms phosphomolybdic acid, which, in turn, is reduced by the addition of 1-amino-2-napthol-4-sulphonic acid (ANSA) to produce a blue-green colour whose intensity is directly proportional to the amount of phosphorus.

Reagents

2.5% acid ammonium molybdate : Appendix.
1-amino-2-napthol-4-sulphonic acid : Appendix.
(ANSA) reagent

Phosphorus standard stock solution : 439.4 mg of KH₂PO₄ dissolved in 100 ml of d.w.
(1 mg Pi/ml)

Working solution : Diluted 1 ml of stock solution in 100 ml of d.w.
(10 μg Pi/ml)

Procedure

After the final stage, namely addition of 10% TCA to mixture, the contents were centrifuged at 1,500 xg for 10 minutes. 1 ml aliquot was mixed with 1 ml of 2.5% ammonium molybdate solution. 0.5 ml of ANSA solution was added and the contents were thoroughly mixed and were kept at 37°C for 5 minutes (or stand at room temperature for 45 min.). At least 2 ml of distilled water was added after incubation time and intensity (OD) of blue-green colour thus developed was read at 595 nm.

Phosphorus standards (1-5 μg Pi/ml) and blank were also run simultaneously.

Adenosine Triphosphatase (ATPase) (EC 3.6.1.3)

Method adopted: with minor modifications:
Mg²⁺-ATPase – Kielley (1955).
Principle

During the hydrolysis of ATP, inorganic phosphorus (Pi) is liberated which can be quantitated biochemically.

\[
\text{ATP} \xrightarrow{\text{ATPase}} \text{ADP} + \text{Pi}
\]

1. Na\(^+\)-K\(^+\)-ATPase assay was performed under two conditions:
   (i) In the presence of Ca\(^{2+}\), Mg\(^{2+}\), Na\(^+\) and K\(^+\) (total ATPase), and
   (ii) In the presence of Ca\(^{2+}\), Mg\(^{2+}\), Na\(^+\), K\(^+\) and Ouabain (Ouabain-insensitive ATPase).

The Na\(^+\)-K\(^+\) stimulated activity was obtained by subtracting the ouabain-insensitive ATPase from the total ATPase activity.

2. Ca\(^{2+}\)-dependent ATPase was also assayed under two conditions:
   (i) In the presence of Ca\(^{2+}\), Mg\(^{2+}\), Na\(^+\) and K\(^+\) (total ATPase), and
   (ii) In the presence of Ca\(^{2+}\), Mg\(^{2+}\), Na\(^+\), K\(^+\) and EGTA (Ca\(^{2+}\)-independent ATPase).

Ca\(^{2+}\)-dependent ATPase was then calculated by subtracting the Ca\(^{2+}\)-independent ATPase from the total ATPase activity.

Reagents

- 75 mM Tris-HCl buffer (pH 7.2)
- 0.2 M Tris-HCl buffer (pH 7.6)
- 75 mM MgCl\(_2\)
- 120 mM NaCl
- 200 mM KCl
- 2.5 M KCl
- 100 mM ATP
- 2.5 M Perchloric acid
- 10 mM Ouabain
- 1.5 mM EGTA
- 5 mM MgCl\(_2\)
- TCA (10%)
Procedure

The general procedure without any drug treatments and/or antioxidant supplementation was as follows:

0.9 ml of an assay mixture containing 0.4 ml of 75 mM Tris-HCl buffer (pH 7.2); 0.1 ml of 75 mM MgCl₂; 0.1 ml of 120 mM NaCl; 0.1 ml of 200 mM KCl and 0.1 ml of 50 mM ATP plus 0.1 ml of distilled water (total ATPase)/10 mM Ouabain (Ouabain-insensitive ATPase)/ 1.5 mM EGTA (Ca²⁺-independent ATPase) was taken in each test tube and incubated for 5 min at 37°C. 0.1 ml of spermatozoal membrane fraction was added to each tube. The tubes were stirred and incubated for 20 min at 37°C. The reaction was terminated by the addition of 0.25 ml of 2.5 M PCA. The tubes were immersed in ice bath for at least 20 min and then 0.25 ml of 2.5 M KCl was added, while the tubes still immersed in ice for another 20 minutes and then centrifuged at 3,000 rpm for 5 minutes to obtain a clear supernatant. The inorganic phosphorus (Pi) thus liberated in the supernatant, was estimated by the method of Fiske and Subbarow (1925) as mentioned earlier.

The general procedure for assaying Mg²⁺-ATPase activity was as follows:

To 0.5 ml of 0.2M Tris-HCl buffer (pH 7.6) and 0.1 ml of 5 mM MgCl₂, 0.1 ml of 50 mM ATP was added and the mixture was preincubated at 37°C for 5 minutes. 0.1 ml of spermatozoal membrane fraction plus 0.2 ml of distilled water were added and then the samples were incubated at 37°C for 30 min. 1 ml of 10% TCA was added to stop the reaction and precipitate the proteins, and kept in fridge for 15 min. The tubes were centrifuged at 3,000 rpm for 5 minutes and then the inorganic phosphorus (Pi), thus liberated in the supernatant, was estimated by the method of Fiske and Subbarow (1925). Various substrate kinetics were performed using different ATP concentrations ranging from 15 to 75 mM in the control group as well as in drug-treated ones and also in drug-treated-antioxidant supplemented series.
Preparation of spermatozoal plasma membrane fraction

Method adopted: Rufo et al. (1984), with minor modifications

Reagents

<table>
<thead>
<tr>
<th>Buffer I (pH 7.4)</th>
<th>0.25 M Sucrose</th>
<th>–</th>
<th>8.56 g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.20 mM MgCl$_2$</td>
<td>–</td>
<td>4.07 mg</td>
</tr>
<tr>
<td></td>
<td>10 mM MOPS</td>
<td>–</td>
<td>0.231 g</td>
</tr>
<tr>
<td>Buffer II (pH 7.4)</td>
<td>0.25 M Sucrose</td>
<td>–</td>
<td>8.56 g</td>
</tr>
<tr>
<td></td>
<td>0.13 mM MgCl$_2$</td>
<td>–</td>
<td>2.64 mg</td>
</tr>
<tr>
<td></td>
<td>10 mM EDTA</td>
<td>–</td>
<td>37.22 mg</td>
</tr>
<tr>
<td></td>
<td>10 mM MOPS</td>
<td>–</td>
<td>0.231 g</td>
</tr>
<tr>
<td>Buffer III (pH 7.4)</td>
<td>0.2 M Sucrose</td>
<td>–</td>
<td>8.56 g</td>
</tr>
<tr>
<td></td>
<td>10 mM MOPS</td>
<td>–</td>
<td>0.231 g</td>
</tr>
</tbody>
</table>

The chemicals of each buffer were dissolved in 100 ml of d.w. and pH adjusted by tris solution.

Procedure

The fresh and well liquefied human ejaculated spermatozoa were centrifuged in cold (4°C) at 500 xg for 10 minutes. The resulting supernatant containing the seminal plasma was discarded while the pellet so obtained was suspended in buffer I and washed twice in the same buffer. The pellet was then suspended in the buffer II and homogenized using a Potter Elvehjæm homogenizer. The suspension was then kept at room temperature for 30 min. This was followed by instant chilling of the sample in an ice-ethanol bath in which it was sonicated at low setting three times for 10 seconds each, using a B. Braun’s Labsonic 2000 sonicator with a microtip probe. The resulting suspension was centrifuged at 500 xg for 10 min. The supernatant was collected and the pellet was washed two times, each time the supernatant was retained and then collectively centrifuged at 100,000 xg for 1 hour. The resulting pellet was washed twice in buffer III and was finally suspended in a minimum amount of the same buffer. This pellet contained the spermatozoal plasma membrane fraction and was used for the various works in assessing the ATPase activities under different conditions.
5'-Nucleotidase (EC 3.1.3.5)

**Method adopted**: Huang and Keenan (1972)

**Principle**

\[ 5'-\text{Ribonucleotide} + \text{H}_2\text{O} \rightarrow \text{Ribonucleoside} + \text{orthophosphate} \]

**Reagents**

- 150 mM Tris-HCl buffer (pH 7.5) : 2.37 g in 100 ml of d.w.
- 20 mM AMP : 35.65 mg in 2 ml of d.w.
- 10% TCA solution : 10 g TCA in 100 ml of d.w.

**Procedure**

To 0.8 ml of assay mixture that contained 0.4 ml of 150 mM Tris-HCl buffer (pH 7.5), 0.2 ml of 20 mM AMP (substrate) and 0.2 ml of d.w., 0.2 ml of spermatozoal membrane fraction was added. To initiate the reaction, the mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 2 ml of chilled 10% TCA solution. The tubes were centrifuged at 1,500 xg for 10 minutes and the inorganic phosphorous thus liberated in the supernatant was estimated by the method of Fiske and Subbarow (1925).

Glucose-6-phosphate Isomerase (D-glucose-6-phosphate Ketol-isomerase (EC 5.3.1.9)

**Method adopted**: Slein (1954)

**Principle**

Hydrogen atom in the CH₂-OH group of glucose-6-phosphate is removed by the enzyme.

\[ \text{D-Glucose-6-phosphate} \xrightarrow{\text{enz.}} \text{D-fructose-6-phosphate} \]

**Reagents**

Substrate : 0.1 M monosodium-salt of glucose-6-phosphate ... 28.4 mg dissolved in 1 ml of d.w.
Materials & Methods

0.1 M Tris-HCl buffer (pH 9.0) : Appendix.
0.1% Alcoholic resorcinol : Added 1 g thiourea and 250 mg resorcinol in 100 ml of glacial acetic acid.
30% HCl solution : Took 21.53 ml of conc. HCl and dissolved in 100 ml of d.w.
Standard (fructose) (10-90 µg/ml) : Dissolved 10 mg of fructose in 100 ml of d.w.

Procedure

The assay mixture containing 0.5 ml Tris-HCl buffer (pH 9.0); 0.1 ml of 0.1 M mono-sodium salt of glucose-6-phosphate (substrate); 0.1 ml of spermatozoal suspension was treated with 0.1 ml of nicotine (0.5 and 1 mM) and/or caffeine (5-9 mM), along with or without antioxidant supplementation. The total volume of assay mixture was 1 ml. The above mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 3.5 ml of 30% HCl and 1 ml of 0.1% alcoholic resorcinol. The reaction mixture was heated for 10 min at 80°C till pinkish colour developed, and then cooled at room temperature. The tubes were centrifuged at 1,500 xg for 10 min. The intensity of colour developed in supernatant was read at 520 nm. The unit of enzyme activity was defined as that amount which causes a change in optical density due to fructose-6-phosphate in 30 minutes of isomerization at 37°C.

Hexokinase (Glucsoe-ATP Phosphotransferase) (EC 2.7.1.1)

Method adopted: Crane and Sols (1955)

Principle

\[
\text{D-Hexose} + \text{ATP} \xrightarrow{\text{enz.}} \text{D-Hexose-6-Phosphate} + \text{Pi} + \text{ADP}.
\]

\[
\text{Glucose-1-Phosphate} + \text{ATP} \xrightarrow{\text{enz.}} \text{Glucose-6-Phosphate} + \text{Pi} + \text{ADP}.
\]

\[
\text{Glucose-6-Phosphate} \rightarrow \text{To all tissues, citric acid and glycolytic pathways.}
\]
Reagents

Substrate : 0.1 M glucose solution ... 17.1 mg in 5 ml of d.w.
ATP-Mg salt solution (pH 7.0) : 88 mg sodium salt of ATP (0.075 M) and 40.5 mg MgCl₂ (0.04M) in 5 ml of d.w.
5% TCA : 5 g TCA in 100 ml of d.w.
Histidine-Tris-EDTA buffer : Appendix.
Standard (KH₂PO₄) : 439.4 mg of KH₂PO₄ in 100 ml of d.w.
Stock solution (1 mg Pi/ml) : 1 ml of stock solution in 100 ml of d.w.
Working solution (10μg Pi/ml) : 0.1 M glucose solution ...

Procedure

The assay mixture containing 0.2 ml of ATP-Mg solution (pH 7.0); 0.2 ml of Histidine-Tris-EDTA buffer; 0.2 ml of 0.1 M glucose solution (substrate), was treated with nicotine (0.5 and 1 mM) and/or caffeine (5,7 and 9 mM), also with antioxidant supplementation. In the control case, instead of drug and antioxidant additions, an equal amount of vehicle of the drug was added. The mixture was warmed at 30°C for 3 minutes. 0.1 ml of spermatozoal suspension was then added. The tubes were mixed well and incubated at 37°C for 30 minutes. The resultant reaction was terminated by adding 0.1 ml of chilled 5% trichloroacetic acid (TCA). The samples were then centrifuged at 1,500 xg for 10 minutes, 1 ml of supernatant was taken out and the amount of Pi liberated was quantitated by the method of Fiske and Subbarow (1925), as described earlier. The intensity of the colour developed in tubes was read at 595 nm.

Lactate Dehydrogenase (LDH) (Lactotase) (EC 1.1.1.28)

Method adopted: Wooton (1964)

Principle

Sodium lactate + NAD⁺ → Sodium pyruvate + NADH + H⁺
Reagents

Buffered substrate : Appendix.
Dinitro phenyl hydrazine (DNPH) solution : Appendix.
NAD+ solution : 5 mg of NAD in 1 ml of d.w. (Freshly used).
0.4 N NaOH solution : 1.6 g of NaOH in 100 ml of d.w.
Sodium pyruvate (standard) : 10 mg of sodium pyruvate in 100 ml of d.w. (ranging from 10 to 50 µg/ml).

Procedure

An assay mixture containing 0.4 ml of buffered substrate; 0.2 ml of freshly prepared NAD+ solution and 0.1 ml of spermatozoal suspension was treated with nicotine and caffeine in different concentrations as well as supplemented with antioxidants. The suitable blank was also run simultaneously. The above mixture was then incubated for 30 min at 37°C. The tubes were shaken and 1 ml of DNPH solution was added and tubes incubated again for 20 min at 37°C, and 2 ml of 0.4 N NaOH solution added. The brownish colour was developed at this stage. After 20 minutes the optical density (OD) of colour in the tubes was read at 440 nm.

Glucose-6-Phosphatase (D-glucose-6-phosphate Phosphohydrolase) (EC 3.1.3.9)

Method adopted: Swanson (1955)

Principle

Glucose-6-Phosphate $\xrightarrow{\text{enz.}}$ Glucose + Pi.

Pi + Ammonium molybdate $\xrightarrow{\text{Reduction}}$ molybdenum $\xrightarrow{}$ blue-green colour

Reagents

0.1 M Citrate buffer : Appendix.
(pH 6.2)
Materials & Methods

Substrate solution: 25 mM solution of glucose-6-phosphate ..... 405.5 mg in 100 ml of citrate buffer (pH 6.2).

10% TCA: 10 g of TCA in 100 ml of d.w. (chilled)

Phosphorus standard

Stock solution (1 mg Pi/ml): 439.4 mg of KH₂PO₄ in 100 ml of d.w.

Working solution (10 pg Pi/ml): 1 ml of stock solution in 100 ml of d.w. μg Pi/ml

Procedure

The assay mixture containing 0.4 ml of 0.1 M citrate buffer (pH 6.2); 0.1 ml of substrate (25 mM solution of glucose-6-phosphate), and 0.1 ml of spermatozoal suspension was treated or not with nicotine (0.5 and 1 mM) and/or caffeine (5-9 mM) and also supplemented with appropriate amount of antioxidants (single/ in combination). The blank tubes were also run simultaneously. The mixtures were mixed well and incubated at 37°C for 30 minutes. The reaction was terminated by adding 1 ml of 10% chilled TCA. The tubes were then centrifuged and the amount of Pi liberated in 1 ml of supernatant was calculated according to the method of Fiske and Subbarow (1925), as explained earlier. The intensity of the developed colour was read at 595 nm.

Glucose-6-Phosphate Dehydrogenase (G-6-PDH) (EC 1.1.1.49)

Method adopted: Julian and Reithal (1961)

Principle

Glucose-6-phosphate enz. → NADPH+H⁺+6-Phosphogluconic acid.

(Substrate) NADP

The change in optical density at 340 nm per unit time gave the measure of G6PDH activity in the media.

Reagents

0.1 M Triethanolamine: 1.86 g triethanolamine hydrochloride in buffer (pH 7.6) 100 ml of d.w. – adjusted pH with 1 N NaOH solution.
Materials & Methods

MgCl₂ solution : 1g MgCl₂ in 50 ml of d.w.
35 μM glucose-6-phosphate : 10 mg in 1 ml of d.w.
0.110 mM NADP solution : 10 mg of NADP in 1 ml of d.w.

Procedure

The spermatozoal homogenate in triethanolamine buffer was centrifuged at 3,000 xg for 10 minutes and the supernatant was taken out as spermatozoal sample. The assay mixture containing 2.1 ml of 0.1 M triethanolamine buffer (pH 7.6); 0.3 ml of MgCl₂ solution; 0.1ml of glucose-6-phosphate (35μM) solution; 0.1 ml of 0.110 mM solution of NADP was treated with nicotine and/or caffeine in various concentrations or not (untreated samples) and also supplemented with antioxidants or not (unsupplemented samples). This mixture was preincubated at 37°C for 2-3 minutes. To start the reaction 0.1 ml of spermatozoal sample in triethanolamine buffer was added to each tube and shaken immediately. The change in the absorbance of mixture (ΔA) was recorded for 5 minutes at 340 nm. The extinction coefficient of NADPH at 340 nm was taken as 6.22 mM⁻¹ cm⁻¹. The unit of enzyme activity was considered as mmol NADPH formed, mg prot⁻¹, min⁻¹.

Motility Test

The number of motile and non-motile spermatozoa were counted respectively at 0 hour and after interval of 10 minutes each for next 2 hours in the untreated-unsupplemented series (control) and test samples incubated at 37°C. To 1 ml of spermatozoal suspension (80x 10⁶ cells / ml) nicotine (0.5 and 1mM) and caffeine (5,7 and 9 mM) were added. The control samples received an equal amount of suitable vehicle i.e., 0.2 M phosphate buffered saline (PBS). Ascorbic acid, glutathione and trolox were supplemented to the treated and untreated spermatozoal samples accordingly. The microscopic fields were scanned systematically according to the WHO laboratory manual (1992). The number of sperm actively moving forward and was counted. The final results...
were expressed as percent motile sperm cells in relation to the total sperm cells.

**Scanning Electron Microscopic Studies**

**Method adopted:** Baccetti (1975), with minor modulation

**Reagents**

- 0.2 M Phosphate buffer (pH 7.0) : Appendix.
- 3% Glutaraldehyde solution : 3 ml of 25% glutaraldehyde was diluted with phosphate buffer (pH 7.2) and the total volume was raised to 25ml.

**Procedure**

The reconstituted spermatozoal samples were incubated for 30 min at 37°C with 1 mM nicotine and/or 9 mM caffeine treatments, supplemented or not with antioxidants. Suitable untreated-unsupplemented series (controls) were also incubated simultaneously. 3% glutaraldehyde solution was added (1:20 v/v), and the samples were mixed well and left for final fixation at room temperature for 30 minutes. These were then centrifuged at 500 xg for 10 minutes and the resulting pellets were washed 3-4 times with triple distilled water, each 5 min. Finally, a small drop of each sample was put on a separate stub. Air dried samples were sputter-coated with gold-palladium (1.2 KV for 3 min) and scanned under a JEOL, 2601 scanning electron microscope (SEM) housed at the Regional Sophisticated Instrumentation Centre (RSIC), at the Panjab University Campus, Chandigarh.

**Single Cell Gel Electrophoresis (Comet) Assay**

**Method adopted:** Singh *et al.* (1988)

Estimation of genetic instability by direct quantitation of DNA damage and repair is an important aspect of studies on mutagenesis and carcinogenesis. Different methods have been introduced from time to time in order to meet this need. The single cell gel electrophoresis (SCGE) is a new, simple method. It is also sensitive and amenable in order to detect the
Materials & Methods

presence of DNA strand breaks and alkali labile damages in the individual cells.

Principle

In nucleus of the cell, relatively fragile DNA is present in the form of compactly arranged supercoiled loops. One nick produced by an clastogenic compound is enough to uncoil the DNA loops from the tightly packed core. The DNA fragments so produced migrate towards the anode, at a rate inversely proportional to the size of the fragment during electrophoresis. Consequently, each cell with damaged DNA gives the appearance of a comet. Hence, this assay is known as comet assay.

Reagents

1. **0.2 M Phosphate buffered saline (pH 7.2)**: Appendix.

2. **Lysing solution**

   To make 1000 ml of lysing solution the following salts were taken:
   
   - 2.5 M NaCl – 146.1 g
   - 100 mM EDTA – 37.2 g
   - 10 mM Tris (Trizma base)– 1.2 g
   - 10% DMSO – 100 ml
   - 1% Triton X-100 – 50 ml

   All the above salts were added to 700 ml of distilled water in a bottle and shaken them well so that all the ingredients were dissolved. Approximately 8 g of NaOH pellet was added and allowed the mixture to dissolve for 20 minutes. Adjusted the pH at 10 by adding concentrated HCl, the mixture being stored at room temperature. For final lysing solution 50 ml of freshly prepared 1% Triton X-100 (990 ml d.w. + 10 ml Triton X-100) and 100 ml of 10% DMSO were added and this formed the 1000 ml of lysing solution. The solution was then filtered and kept in the refrigerator for 30-60 min prior to slide preparation.

3. **Electrophoresis buffer/Alkaline buffer**

   (300mM NaOH/1mM EDTA)
Stock solution

A : 10 N NaOH – Added 200 g of NaOH in 500 ml of d.w. and mixed them thoroughly (shelf life two weeks).

B : 200 mM EDTA – Added 14.89 g of EDTA in 300 ml of d.w (pH 10.0).

It was shaken well and stored, both the stock solutions A and B at room temperature.

Working solution (IX buffer) (pH 12.1)

For making 1000 ml of electrophoresis buffer solution taken 30 ml of stock solution A and 5 ml of stock solution B. To this 965 ml of distilled water was added and the flask was shaken to mix all the solutions. This working solution is prepared immediately before running the electrophoresis.

4. Neutralizing buffer

48.5 g Tris (0.4 M) was added to 800 ml of distilled water and adjusted the pH to 7.5 with HCl solution. The total volume was made 1000 ml with d.w. Then stored this buffer at room temperature.

5. Staining solution (Acridine orange)

85 mg of acridine orange was added to 100 ml of distilled water. Staining solution is stable for several hours at room temperature and for 1-2 days when refrigerated. It is made fresh and filtered prior to use.

6. Agarose gel preparation

a) Normal melting point agarose (1.0%)

For preparing 5 ml of agarose solution 50 mg of agarose was added to 5 ml of PBS (pH 7.2). Heated this mixture up to the boiling point, then kept at room temperature.

b) Low melting point agarose (1.0%)

For preparing 5 ml of low melting point agarose solution 50 mg of agarose was added to 5 ml of PBS (pH 7.2). Heated this mixture up to the boiling point and kept at 37°C temperature.
Procedure

a) Preparation of slides for comet assay

Put single layer of normal agarose (200-300 μl) on the rough surface of a frosted microscopic slide and immediately placed coverslip for the proper spreading of the gel. Allowed the agarose to solidify for 3-4 minutes at room temperature. Then gently removed the coverslip and added 80 μl of low melting point agarose solution mixed with 20 μl of spermatozoal sample. Replaced the coverslip and allowed the agarose to solidify for 3-4 minutes in a refrigerator. Removed the coverslip and added the third layer of the low melting point agarose solution (100 μl) on the slide, allowed it to harden in a refrigerator (3-4 min).

b) Lysis

Gently removed the coverslip after solidification of the agarose. Put the slide into a couplin jar having cold, freshly prepared lysing solution in it and left it for one hour at 4°C in a refrigerator. This treatment lyses the nuclear and the cell walls and permits DNA unfoldings (Tice et al., 1990). Steps a & b were performed under dimmed yellow lights to prevent DNA damages.

c) Alkali treatment and electrophoresis

Removed the slides from the lysing solution after one hour. Placed them side by side near the anode end of the horizontal gel electrophoresis unit which was filled with alkaline buffer until liquid level completely covered the slides. Avoid forming bubbles over the agarose. Slides were allowed to sit in alkaline buffer for 20-60 min prior to turning on the set. The longer the exposure to alkali, the greater the expression of alkali-labile damages. Then turned on the power supply which was maintained at 25 volts and adjusted the current at 300 milliamperes. The current was passed for about 20 min.

d) Neutralization and staining

Take 3 couplin jars filled with neutralizing buffer solution. Gently removed the slides from the electrophoresis unit by switching off the power
and placed them into the couplin jar. Keeping in each jar for 5 minutes, so that the alkaline buffer is completely washed off (repeated two more times).

After neutralization drained the slides completely and added 60 µl of acridine orange on them and covered them with the coverslips. Then sealed the slides with nail polish.

e) **Observation of DNA damage**

The DNA damage was visualized under a fluorescent microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. Comet assay was used to detect the damaged cells, so the tailed cells showed the mutagenicity/genotoxicity. The length of the tail depends upon the concentration of compound/drug which was used. DNA quality in human spermatozoa was evaluated by measuring 50 sperm cells on one slide. At least 3 slides were prepared for each treatment. The percentage of sperm cells with damaged DNA was noted. The categories of DNA damages were undamaged (no migration) and damaged (migrated) DNA. The appearance of comet in the treated sperm cells was as follows:

*Nucleus of normal sperm cell*

```
L - B = 0
```

*Nucleus of treated sperm cell (comet)*

```
L - B = ?
L = length
B = breadth
```
Statistical Analysis


All the results were expressed as mean ± SD. The data was statistically analysed by using the Student’s t-test to establish the validity of the investigation:

a) Standard deviation (SD) = \[ \sqrt{\frac{\sum (\text{Individual value} - \text{Mean})^2}{n - 1}} \]

b) Standard error (SE) = \[ \frac{\text{Standard deviation}}{\sqrt{n - 1}} \]

c) Student’s t-test (t) = \[ \frac{\text{Mean}_1 - \text{Mean}_2}{\sqrt{(SE_1)^2 + (SE_2)^2}} \]

d) Degree of freedom (d.f.) = \[ n_1 + n_2 - 2 \]

where n: Number of observations; Mean\(_1\): Mean value of control data; Mean\(_2\): Mean value of treated data; SE\(_1\): Standard error of the mean of control data; SE\(_2\): Standard error of the mean of experimental data.

Significance

The probability for obtaining ‘t’ value for a given degree of freedom (d.f.) was determined by comparing the ‘t’ values with probability ‘p’. The values were considered significant to the following convention:

p>0.05: Non-significant, p<0.05: Significant; p<0.01: Significant; p<0.001: Highly significant.

Treatment of enzyme kinetic data

The Michaelis-Menten hypothesis (White et al., 1973)

The most important feature of this hypothesis is the assumption that an intermediate enzyme-substrate complex is formed in an enzymatic reaction. It is further assumed that the rate of conversion of the substrate to the product(s) of the reaction is determined by the rate of conversion of the enzyme-substrate complex to reaction products and the enzyme. The following scheme may be written to illustrate this concept:
Materials & Methods

E + S ←——> ES ———> E + P
Enzyme  Substrate  Enzyme-substrate complex  Product

Thus, the rate of product formation depends on the concentration of ES. If the rate of formation of P depended directly on substrate [S], then at constant enzyme [E] a linear relationship could be expected between velocity and the concentration of S. Since this is not obtained, Michaelis and Menten proposed the following explanation; it being assumed that only a single substrate and a single product is formed. It is also assumed that the process proceeds essentially to completion and that the concentration of the substrate is much greater than that of the enzyme in the system.

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} P + E \quad \ldots(1)
\]

where \( k_1, k_{-1}, \) and \( k_2 \) are the respective velocity constants of the three assumed processes. For the rate of formation of ES (\( v_f \)) we may write

\[
v_f = k_1 ([E_i] - [ES]) [S] \quad \ldots(2)
\]

where \( E_i \) is the total enzyme and \([E_i]-[ES]\) is the concentration of the uncombined enzyme. This states that the rate of formation of ES, which is in fact \( v_f \), is proportional to the concentration of uncombined enzyme and substrate. The rate of disappearance of ES, \( v_d \), is then

\[
v_d = k_{-1} [ES] + k_2 [ES] \quad \ldots(3)
\]

since ES can disappear to give the initial reactants (\( k_{-1}\)) or by the formation of products (\( k_2 \)) (see equation 1).

When the rates of formation and disappearance of ES are equal, i.e. when \( v_f \) (equation 2) equals \( v_d \) (equation 3), then equation 4 describes the steady state.

\[
k_1 ([E_i]-[ES]) [S] = k_{-1} [ES] + k_2 [ES] \quad \ldots(4)
\]
The terms may be rearranged to give
\[
\frac{[S] (\lbrack E \rbrack - [ES])}{[ES]} = \frac{k_1 + k_2}{k_1} = k_m \quad \ldots \ldots \ldots (5)
\]

The term containing the three velocity constants is \( k_m \), the Michaelis-Menten constant, and is a particularly useful parameter, characteristic of each enzyme.

The relationships among the substrate concentration, the enzyme concentration and the velocity of the enzyme-catalyzed reaction can be developed in the following manner. From equation 5, by rearrangement to solve for \([ES]\), the steady-state concentration of the enzyme-substrate complex is obtained,
\[
\frac{[E] [S]}{k_m + [S]} \quad \ldots \ldots \ldots (6)
\]

Because, it is not generally feasible to measure the concentration of ES, therefore in order to ascertain \( k_m \), it is necessary to derive an expression that relates \( k_m \) to the readily measured parameters \([E]\) and \([S]\). Advantage is taken of the relationship.

\[
v = k_2 [ES] \quad \ldots \ldots \ldots (7)
\]
where \( v \) is the observed initial velocity. When the substrate concentration is made so high in relation to the enzyme concentration that essentially all the enzyme is present as ES, then the velocity of the reaction is maximal, and this velocity \( V_{\text{max}} \), has the value
\[
V_{\text{max}} = k_2 [E] \quad \ldots \ldots \ldots (8)
\]
By substituting for ES in equation 7, its value in equation 6, and dividing equation 7 by equation 8, there is obtained the desired expression:
\[
v = \frac{V_{\text{max}} [S]}{k_m + [S]} \quad \text{or} \quad k_m = \frac{V_{\text{max}}}{[S]} \left( \frac{V_{\text{max}}}{v} - 1 \right) \quad \text{or} \quad v = \frac{V_{\text{max}}}{1 + k_m /[S]} \quad \ldots \ldots \ldots (9)
\]
This is the Henri-Michaelis-Menten equation.
For experimental determination of $k_m$, the velocity of the reaction (relative activity of the enzyme) is measured as a function of substrate concentration. These experimentally determined values may be plotted against one another as indicated in Fig. d.

![Graph showing effect of substrate on enzyme reaction](image)

**Fig. d:** Effect of substrate on enzyme reaction (Adopted from White et al., 1973)

By rearranging equation 9, it will be seen that

$$v = \frac{1}{V_{max}} \frac{1}{1 + k_m/[S]}$$

Hence, when $v = \frac{1}{2} V_{max}$,

$$\frac{1}{1 + k_m/[S]} = \frac{1}{2} \quad \text{and} \quad k_m = [S]$$

Thus, $k_m$ is equal to that concentration of the substrate that gives half the numerical maximal velocity, $V_{max}$.

Because the $v$ versus $[S]$ curve is a hyperbola it is rather difficult to determine $V_{max}$, and hence, the $[S]$ that yields $\frac{1}{2} V_{max}$ (i.e. $k_m$) is preferred.
Materials & Methods

Relative velocity \((v)\)

Substrate concentration \([S]\)

To facilitate the determination of the kinetic constants, the data are usually plotted in one of the linear \((y=mx+b)\) forms. One of the most commonly used methods to determine the \(k_m\) depends on rearrangement of equations (9) to give the following form:

\[
\frac{[S]}{v} = \frac{k_m}{V_{max}} + \frac{1}{V_{max}} \quad \text{ ...(10)}
\]

A plot of \([S]/v\) versus \([S]\) gives a straight line as described by Hanes-Woolf. The intercept of the line on the \([S]/v\) axis is \(k_m/V_{max}\) and the slope is \(1/V_{max}\) (Fig.e). Thus, \(k_m\) can be calculated from the slope and the \(V_{max}\) from the intercept. Three additional methods of plotting kinetic data are also shown in Figs.f-h. These are the Lineweaver-Burk, Woolf-Augustinsson-Hofstee and Eadie-Scatchard, respectively (Segel, 1976).

Fig. e

\([S]/v\)  
Slope = \(1/V_{max}\)

\(k_m/V_{max}\)

[S]

Fig. f

\(1/v\)  
Slope = \(k_m/V_{max}\)

\(1/V_{max}\)

\(1/[S]\)
Fig. 6-h: Methods of plotting substrate kinetics data (Adopted from White et al., 1973; Segel, 1976).

The Lineweaver-burk reciprocal plot: $1/v$ versus $1/[S]$, is based on the rearrangement of the Henri-Michaelis-Menten equation as follows:

$$\frac{v}{V_{max}} = \frac{[S]}{K_m + [S]}$$

Inverting:

$$\frac{V_{max}}{v} = \frac{K_m + [S]}{[S]}$$

Cross multiplying:

$$\frac{1}{v} = \frac{k_m + [S]}{V_{max} [S]}$$

Separating terms:

$$\frac{1}{v} = \frac{k_m}{V_{max} [S]} + \frac{[S]}{V_{max} [S]}$$

or

$$\frac{1}{v} = \frac{k_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

...(11)

Here also a plot of $1/v$ versus $1/[S]$ gives a straight line. The intercept of the line on the $1/v$ axis is $1/V_{max}$ and that on the $1/[S]$ axis gives the value of $1/k_m$. 

74
Any substance that reduces the velocity of an enzyme-catalyzed reaction can be considered to be an inhibitor. The inhibiting capacity may follow any one of the following categories (Segel, 1976; Price and Stevens, 1989):

i) Competitive

ii) Non-competitive (Reversible and Irreversible)

iii) Uncompetitive

iv) Linear mixed type

The velocity equation (Henri-Michaelis – Menten eqn.) in the absence of an inhibitor is:

\[
V_o = \frac{[S] \cdot V_{\text{max}}}{k_m + [S]}
\]

### i) Competitive inhibition

A competitive inhibitor is a substance that combines with free enzyme in a manner that prevents substrate binding. That is, the inhibitor and the substrate are mutually exclusive, often because of true competition for the same site. A competitive inhibitor might be a non-metabolizable analog or derivative of the true substrate, or an alternate substrate of the enzyme, of a product of the reaction.

The reaction scheme describing ‘dead-end’ competitive inhibition is:

\[
E + S \xrightleftharpoons[k_i]{k_4} ES \xrightarrow{k_p} E + P
\]

\[
k_i = \frac{[E][I]}{[EI]}
\]

In general, a competitive inhibitor [I] acts only to increase the apparent \(k_m\) for the substrate. As [I] increase, \(k_{\text{m,app}}\) increases. The \(V_{\text{max}}\) remains unchanged, but in the presence of a competitive inhibitor a much greater
substrate concentration is required to attain any fraction of $V_{\text{max}}$. The degree of inhibition caused by a competitive inhibitor depends on $[S]$, $[I]$, $k_m$, and $k_i$. An increase in $[S]$ at constant $[I]$ decreases the degree of inhibition. An increase in $[I]$ at constant $[S]$ increases the degree of inhibition. The lower the value of $K_i$, the greater is the degree of inhibition at any given $[S]$ and $[I]$. $K_i$ is equivalent to the concentration of $I$ that doubles the slope of the $1/v$ versus $1/[S]$ plot.

ii) Non-competitive inhibition

A classical non-competitive inhibitor has no effect on substrate binding and vice versa. $S$ and $I$ bind reversibly, randomly, and independently at different sites. That is, $I$ binds to $E$ and to $ES$; $S$ binds to $E$ and to $EI$. However, the resulting $ESI$ complex is catalytically inactive. $[I]$ might prevent the proper positioning of the catalytic center.

\[
\begin{align*}
E + S & \underset{k_i}{\overset{k_a}{\rightleftharpoons}} ES \\
E + I & \underset{k_i}{\overset{k_p}{\rightleftharpoons}} E + P \\
EI + S & \underset{k_e}{\overset{k_i}{\rightleftharpoons}} ESI
\end{align*}
\]

\[
\begin{align*}
[k_i] &= \frac{[E][I]}{[EI]} = \frac{[E][I]}{[ESI]} \\
[k_a] &= \frac{[E][S]}{[ES]} = \frac{[E][S]}{[ESI]}
\end{align*}
\]

Therefore, at any $[I]$ a portion of the enzyme will remain as the non-productive $ESI$ complex. Consequently, one can predict that the $V_{\text{max}}$ in the presence of a non-competitive inhibitor ($V_{\text{max}}^i$) will be less than $V_{\text{max}}$ observed in the absence of inhibitor. The $k_m$ value will be unchanged because at any inhibitor concentration, the enzymatic forms that can combine with $S$ ($E$ and $EI$) have equal affinities for $S$. The net effect of a non-competitive inhibitor is to make it appear as if less total enzyme is present.
Irreversible inhibition

A substance that combines irreversibly with an enzyme may resemble a non-competitive inhibitor because \( V_{\text{max}} \) is decreased but \( k_m \) remains unchanged. The reactions are:

\[
E + S \xrightarrow{k_s} ES \xrightarrow{k_p} E + P
\]

\[
+ I \xrightarrow{k_i} EI
\]

A plot of \( V_{\text{max}} \) versus amount of enzyme added will distinguish between a reversible and an irreversible non-competitive inhibitor.

(iii) Uncompetitive inhibition

A classical uncompetitive inhibitor is a compound that binds reversibly to the enzyme-substrate complex yielding an inactive ESI complex.

\[
E + S \xrightarrow{k_s} ES \xrightarrow{k_p} E + P
\]

\[
+ I \xrightarrow{k_i} ESI
\]

The equilibria shows that at any \([I]\) and infinitely high substrate concentration the reaction will not drive all of the enzyme to the ES form; some non-productive ESI complex will always be present. Consequently, \( V_{\text{max}} \) in the presence of uncompetitive inhibitor \( (V_{\text{max}}) \) will be lower than \( V_{\text{max}} \) in its absence. The apparent \( k_m \) decreases due to ESI formation. Both \( V_{\text{max}} \) and \( k_m \) are affected by the inhibitor giving rise to parallel lines.

(iv) Linear mixed type inhibition

The scheme for mixed - type inhibition represents both competitive and uncompetitive components, that is:
The velocity equations for the above mentioned enzyme inhibition categories are as follows:

(i) **Competitive**

\[
v_i = \frac{[S] \cdot V_{\text{max}}}{k_m \left[1 + \frac{[I]}{k_i}\right] + [S]}
\]

(ii) **Non-competitive**

\[
v_i = \frac{[S] \cdot V_{\text{max}}}{[S] + k_m \left[1 + \frac{[I]}{k_i}\right]}
\]

(iii) **Uncompetitive**

\[
v_i = \frac{[S] \cdot V_{\text{max}}}{k_m + [S] \left[1 + \frac{[I]}{k_i}\right]}
\]

(iv) **Linear mixed type**

\[
v_i = \frac{[S] \cdot V_{\text{max}}}{k_s \left(1 + \frac{[I]}{k_i}\right) + [S] \left(1 + \frac{[I]}{k_i}\right)}
\]