CHAPTER II

AIM OF THE WORK

The present study was undertaken to investigate the effect of acute whole body exposure to low level of deep x-irradiation on cellular oxidative stress. Production of free radicals and the efficacy of antioxidant defense in liver, brain and testicles of mice were assessed. The relationship between the extent of oxidative stress and the balance between pro-inflammatory and anti-inflammatory cytokines was also considered.

The strategy of protection against cellular damage by whole body deep x-irradiation would be through restoring homeostasis of the disturbed biochemical pathways by affording protection to and preventing damage of essential macromolecules. In this respect, the possible protective effect of treatment with the compatible solute ectoine, which is known to reduce cell stress effects on the molecular level, was evaluated.

CHAPTER III

MATERIALS AND METHODS

Generation of x-rays:

X-rays were generated by allowing an accelerated electron beam to hit a target made of high atomic number material (tungsten). This was achieved in the present study by using a linear medical accelerator (Siemens model Primus) illustrated in Figure (2).

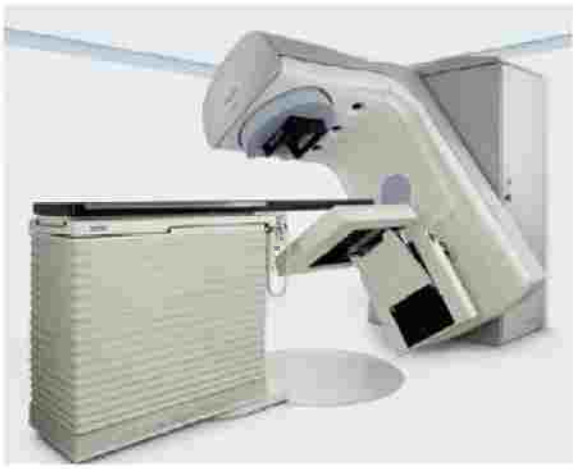

Figure (2): Medical linear accelerator.

The machine is of dual photon energy (6 MV and 15 MV) and multi-energy electrons (6, 9, 12, 16 and 20 MeV). It uses high – frequency electromagnetic waves to accelerate electrons to high energies through linear tube .The main components of linear accelerator are the electron gun, the electron accelerating tube, the beam bending magnet and the source of microwave power. For use of electrons directly in the treatment, the electron beam is bent by electric magnet to hit scattering foil made of stainless steel.

In the present study, the 6MV photon energy was used with a dose rate of 200 monitor units (MU). This results in a dose energy rate of 200 cGy/ minute. Accordingly, irradiation for one minute gives a dose of 2Gy and for three minutes gives a dose of 6 Gy.

Animals:

Male Swiss albino mice, weighing 20-22 g each, were used as the experimental animals. Mice were obtained from the animal house of Medical Research Institute, Alexandria University, and were kept under observation for at least one week prior to study with free access to food and water. All procedures were performed in accordance with regulations of the National Research Council's guide for the care and use of laboratory animals .Animals were divided into the following groups:

Control animals:

Group 1: of six animals served as the negative control and received saline intraperitoneally.

Irradiated animals:

Group 2 : of 12 animals irradiated by a single low dose of 2 Gy. Six (6) animals were sacrificed after 24 hours and the other six (6) were sacrificed after one week.

Group 3 : of 12 animals irradiated by a single high dose of 6 Gy. Six (6) animals were sacrificed after 24 hours and the other six (6) were sacrificed after one week.

Animals in groups 2 and 3 received intraperitoneal injections of saline.

Irradiated treated animals:

Group 4: of 12 animals pretreated with 200 mg/kg of ectoine [78] intraperitoneally and irradiated by a single low dose of 2 Gy. Six (6) animals were sacrificed after 24 hours and the other six (6) after one week. Animals sacrificed after one week received daily doses of 200 mg/kg ectoine.

Group 5: of 12 animals pretreated with 200 mg/kg of ectoine intarperitoneally and irradiated by a single dose of 6 Gy. Six (6) animals were sacrificed after 24 hours and the other six (6) after one week. Animals sacrificed after one week received daily doses of 200 mg/kg ectoine.

Animals were sacrificed by cervical dislocation. Dead animals were decapitated and the bones at the top of the skull were excised to remove the whole brain. The liver and the testicles were also dissected, and the three organs were quickly washed with ice-cold saline. All specimens were properly labeled and kept at -80 ˚C until assayed for the following parameters:

- Interleukin-1β and interleukin-6, representing pro-inflammatory cytokines
- Interleukin-10, representing anti-inflammatory cytokines
- Malondialdehyde (MDA),reduced glutathione (GSH) and oxidized glutathione (GSSG) to evaluate oxidative stress
- Prostaglandin E_2(PGE_2)

Determination of interleukin-6(IL-6):

Quantitative determination of IL-6 was performed in extracts of mice liver, brain and testis tissues using RayBio®Mouse IL-6 ELISA (Enzyme-Linked Immunosorbent Assay) kit [92].

Principle:

This assay employs an antibody specific for mouseIL-6 coated on a 96-well plate. Standards and samples are pipetted into the wells and IL-6 present in a sample is bound to

the wells by the immobilized antibody. The wells are washed and biotinylated anti-mouse IL-6 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin (horseradish peroxidase) is pipetted to the wells. The wells are again washed, a TMB (tetramethylbenzidine) substrate solution is added to the wells and color develops in proportion to the amount of IL-6 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. A standard curve is prepared and IL-6 concentration in the samples is determined.

Reagents:

- IL-6 Microplate of 96 well coated with anti-mouseIL-6
- 25 ml of Wash Buffer Concentrate
- Standards: recombinant mouse IL-6.
- 10 ml Sample Diluent Buffer
- Assay Diluent: 15 ml of 5x concentrated buffer for Detection Antibody and HRP-Streptavidin concentrate diluent.
- Detection Antibody IL-6: biotinylated anti-mouseIL-6
- 200µl HRP-Streptavidin concentrate
- 12 ml of Substrate Reagent (tetramethylbenzidineTMB)
- 8 ml Stop Solution (0.2 M sulfuric acid)

Assay procedure:

1. 100 µl of each standard and sample were added into appropriate wells, covered and incubated for 2.5 hours at room temperature with gentle shaking.
2. The solution was discarded and the wells were washed 4 times with the Wash Buffer. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting.
3. 100 µl of biotinylated antibody were added to each well and incubated for 1 hour at room temperature with gentle shaking. The solution was then discarded and the wash was repeated.
4. 100 µl of prepared Streptavidin solution were added to each well, incubated for 45 minutes at room temperature with gentle shaking followed by discarding the solution and repeating the wash.
5. 100 µl of TMB Substrate Reagent were added to each well, incubated for 30 minutes at room temperature in the dark with gentle shaking.
6. Finally, 50 µl of Stop Solution were added to all wells and the optical density (OD) was determined using a microplate reader at 450 nm immediately.

Calculation of results:

- The absorbance of each standard and sample was read and the zero standard optical density was subtracted.
- The concentration of IL-6 in each sample was estimated from the standard curve constructed by plotting the absorbance of each standard on the y-axis and the concentration on the x-axis Figure (3).

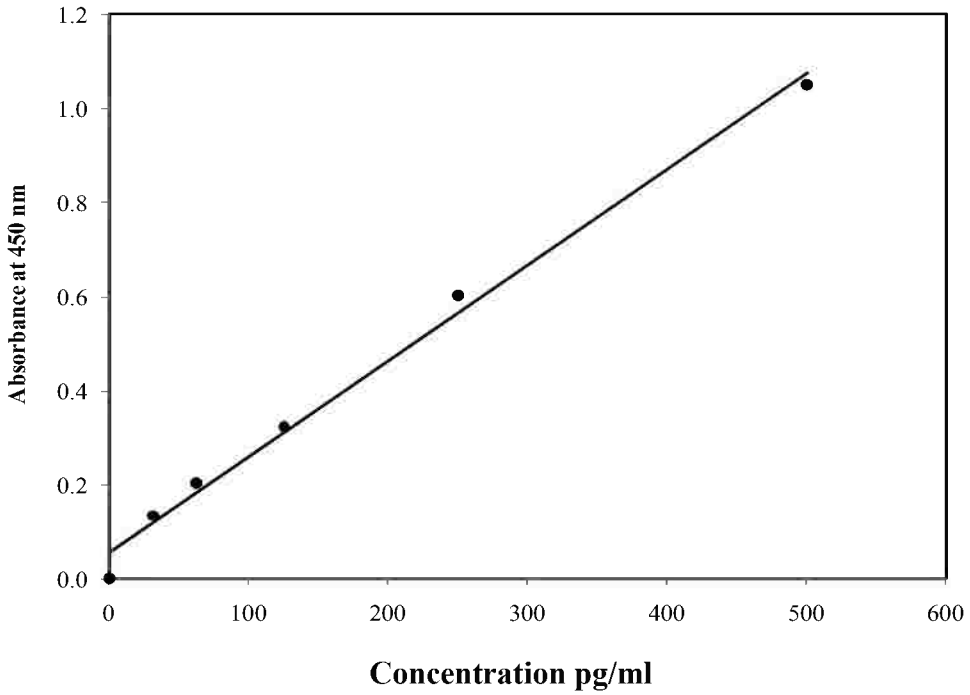

Figure (3): Standard curve for IL-6

Determination of interleukin-1 beta (IL-1β):

Quantitative determination of IL-1β was performed in extracts of mice liver, brain and testis tissues using RayBio® Mouse IL-1β ELISA kit [92].

Principle:

This assay employs the same principle of the assay of IL-6 but using an antibody specific for mouse IL-1β coated on a 96-well plate. The color produced is formed in proportion to the amount of IL-1β present in the mice liver, brain and testis tissues. The intensity of the color is measured at 450 nm. A standard curve is prepared and IL-1β concentration in the samples is determined.

Reagents:

- IL-1β Microplate of 96 well coated with anti-mouse IL-1β
- Wash Buffer Concentrate
- Standards: recombinant mouse IL-1β.
- Sample Diluent Buffer
- Assay Diluent: 15 ml of 5x concentrated buffer for Detection Antibody and HRP-Streptavidin concentrate diluent.
- Detection Antibody IL-1β: biotinylated anti-mouseIL-1β.
- HRP-Streptavidin concentrate
- TMB Substrate Reagent
- Stop Solution (0.2 M sulfuric acid)

Assay procedure:

The same procedure was followed as described in the assay of IL-6:

- 100 µl of each standard and sample were added into appropriate wells, covered and incubated for 2.5 hours at room temperature with gentle shaking.
- 100 µl ofbiotinylated antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking.
- 100 µl of prepared Streptavidin solution was added to each well, incubated for 45 minutes at room temperature with gentle shaking.
- 100 µl of TMB Substrate Reagent was added to each well, incubated for 30 minutes at room temperature in the dark with gentle shaking.
- Finally, 50 µl of Stop Solution was added to each well and the optical density (OD) was determined using a microplate reader at 450 nm immediately.

Calculation of results:

- The absorbance of each standard and sample was read and the zero standard optical density was subtracted.
- The concentration of IL-1β in each sample was extrapolated from the standard curve constructed by plotting the absorbance of each standard on the y-axis and the concentration on the x-axis Figure (4).

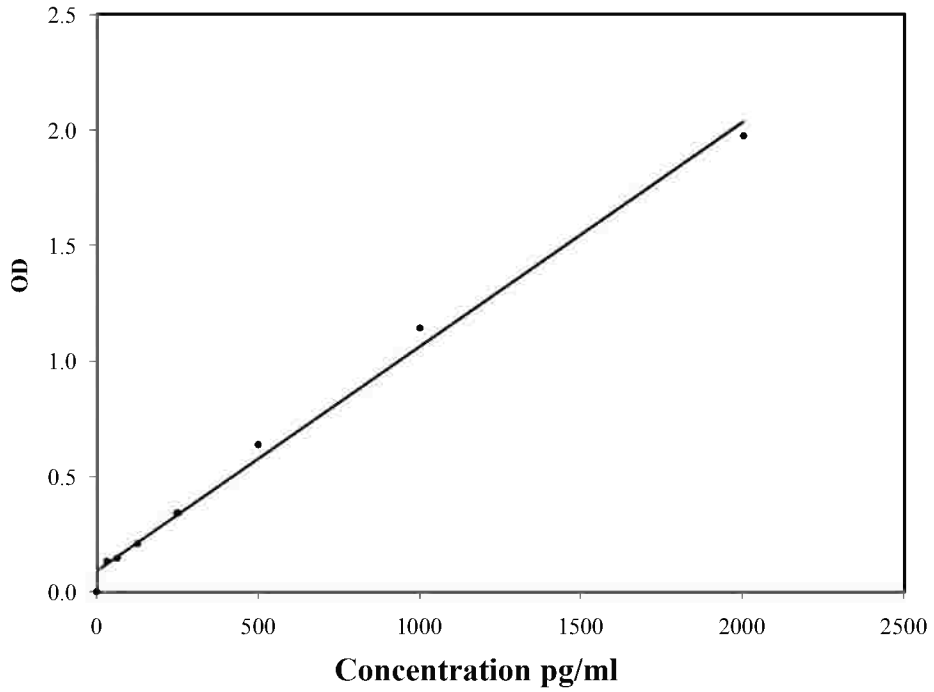

Figure (4): Standard curve for IL-1 Beta

Determination of Interleukin-10

Quantitative determination of IL-10 was performed in extracts of mice liver, brain and testis tissues using RayBio® Mouse IL-10 ELISA kit [92].

Principle:

This assay employs the same principle of the assay of IL-10 but using an antibody specific for mouse IL-10 coated on a 96-well plate. Standards and samples are pipetted into the wells and IL-10 present in a sample is bound to the wells by the immobilized antibody. A color develops in proportion to the amount of IL-10 present in the samples. The intensity of the color is measured at 450 nm.

Reagents:

- IL-10Microplate: 96 well coated with antimouseIL-10
- Wash Buffer Concentrate.
- Standards: recombinant mouse IL-10.
- Assay Diluent: 15 ml of 5x concentrated buffer for Detection Antibody and HRP-Streptavidin concentrate diluent.
- Detection Antibody IL-10: biotinylated anti-mouseIL-10.
- HRP-Streptavidin concentrate
- TMB Substrate Reagent
- Stop Solution(0.2 M sulfuric acid)

Assay procedure:

1. 100 µl of each standard and sample were added into appropriate wells, covered and incubated for 2.5 hours at room temperature with gentle shaking.
2. The solution was discarded and the wells were washed 4 times with Wash Solution.
3. 100 µl of biotinylated antibody were added to each well and incubated for 1 hour at room temperature with gentle shaking. The solution was then discarded and the wash was repeated.
4. 100 µl of prepared Streptavidin solution were added to each well, incubated for 45 minutes at room temperature with gentle shaking followed by discarding the solution and repeating the wash.
5. 100 µl of TMB Substrate Reagent was added to each well, incubated for 30 minutes at room temperature in the dark with gentle shaking.
6. Finally, 50 µl of Stop Solution were added to each well and the optical density was determined using a microplate reader at 450 nm immediately.

Calculation of results:

- The absorbance of each standard and sample was read and the zero standard optical density was subtracted.
- The concentration of IL-10 in each sample was extrapolated from the standard curve constructed by plotting the absorbance of each standard on the y-axis and the concentration on the x-axis Figure (5).

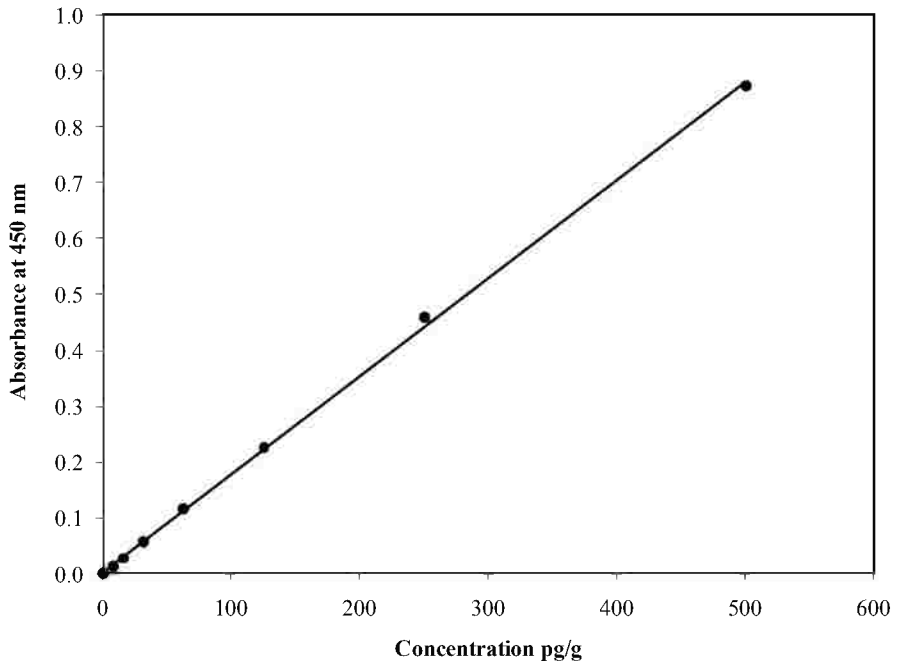

Figure (5): Standard curve for IL-10

Determination of prostaglandin E_2:

Quantitative determination of PGE_2 was performed in extracts of mice liver, brain and testis tissues using Gentaur® Mouse PGF_2 ELISA kit [93].

Principle:

The Mouse PGE_2 polyclonal antibodies are precoated onto 96-well plate. Standard and samples are pipetted into the wells and Mouse PGE2 present in a sample is bound to the wells by the immobilized antibody. The biotinylated detection antibodies are added to the wells and then followed by washing with PBS or TBS buffer. After washing away unbound biotinylated antibody, Avidin-Biotin-Peroxidase Complex is pipetted to the wells. The wells are washed again, a TMB substrate solution is added to the wells and the color changes after adding acidic stop solution. The intensity of the color is proportional to the amount of Mouse PGE_2bound and measured at 450nm.

Reagents:

- Pre-coated Microplate: 96 wells coated with anti-Mouse PGE_2
- Standards: recombinant mouse PGE_2
- Detection Antibody Mouse PGE_2
- Avidin-Biotin-Peroxidase Complex(ABC)
- Sample Diluent Buffer
- Antibody Diluent Buffer
- ABC Diluent Buffer
- TMB color developing reagents
- TMB stop solution
- ELISA Special TBS (Tris Buffered Saline) Diluent

Assay prodcedure:

1- 0.1ml of Mouse PGE_2 standard solutions and samples were added into the precoated 96-well plate.
2- The plate was covered and incubated at 37°C for 90 min, then the contents were discarded and the plate was washed twice with 0.01M PBS (Phosphate buffered saline).
3- 0.1ml of biotinylated anti-Mouse PGE_2 antibody was added into each well and the plate was incubated at 37°C for 60 min then washed three times with 0.01 M PBS.
4- 0.1ml of prepared ABC solution was added into each well and incubation was done at 37°C for 30 min followed by 5 times wash 0.01M PBS.
5- 0.09 ml of prepared TMB color developing agent were added into each well and the plate was incubated at 37°C away from light till shades of blue were seen in the wells with the three-four most concentrated Mouse PGE_2 standard solutions.
6- 0.1ml of prepared TMB stop solution was added into each well. The color changed into yellow immediately.
7- The absorbance was read at 450 nm within 30 min after adding the stop solution.

Calculation of results:

A standard curve was constructed by plotting the O.D. of each standard solution (y-axis) vs. the respective concentration of the standard solution (x-axis). The Mouse PGE_2 concentration of the samples was interpolated from the standard curve .figure (6)

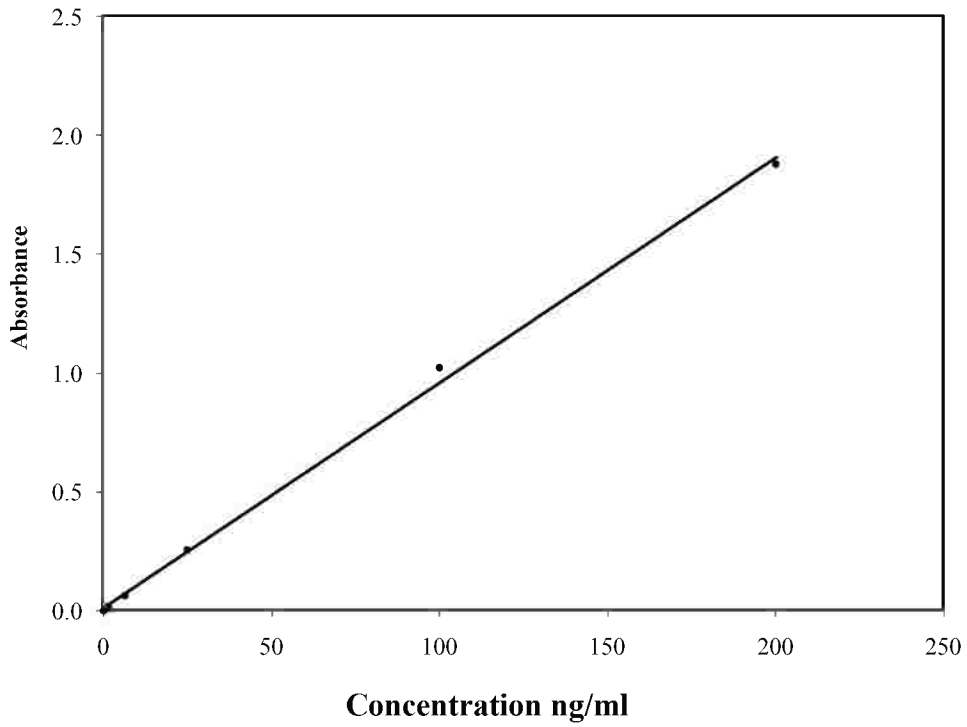

Figure (6): standard curve for Prostaglandine E2

Determination of malondialdehyde (MDA) by thiobarbituric acid reaction:

Reaction principle:

Tissue malondialdehyde was determined by the thiobarbituric acid (TBA) method described by Ohkawa et al [94]. Thiobarbituric acid (TBA) test is most frequently used as an index of lipid peroxidation. It depends on the fact that, when polyunsaturated fatty acids (PUFAs) or esters containing 3 or more double bonds undergo auto-oxidation, a secondary product of lipid peroxidation, which is referred to as malondialdehyde is produced. In this assay, one molecule of malondialdehyde, the most abundant aldehyde product of lipid peroxidation, reacts with two molecules of thiobarbituric acid (TBA) at pH 3.5 to yield a pink chromagen that can be detected spectrophotometerically at 532 nm.

Reagents:

- 1.15% potassium chloride.
- 0.8% TBA in distilled water
- 8.1% sodium dodecyl sulphate (SDS) in distilled water
- 20% acetic acid (pH 3.5) adjusted with 1 N NaOH
- N-butanol
- 1,1, 3,3-tetramethoxypropane (TMP)

Assay procedure:

- Small parts of the liver, brain and testis were weighed and homogenized in 9 volumes of 1.15% KCl to prepare 10% homogenate (w/v).
- An aliquot of 0.1 ml tissue homogenate was added to 0.2 ml of SDS. This was followed by the addition of 1.5 ml of acetic acid and 1.5 ml of aqueous solution of TBA. This mixture was finally made up to 4 ml with distilled water, vortexed and then heated in a water bath at 95 °C for 60 minutes.
- After cooling to room temperature, 1 ml of distilled water and 5 ml of n-butanol were added. This was followed by vigorous shaking and centrifugation at 4000 rpm for 10 minutes.
- The absorbance of the organic layer was read at 532 nm against blank containing 0.1 ml distilled water instead of the sample and treated exactly like the sample.

Calculation

The level of MDA in the samples was determined in nmol/gm tissue from a standard curve made by preparing serial dilutions of TMP (Sigma Chemical Co-USA) in 97% ethanol and treating them exactly like samples, Figure (7).

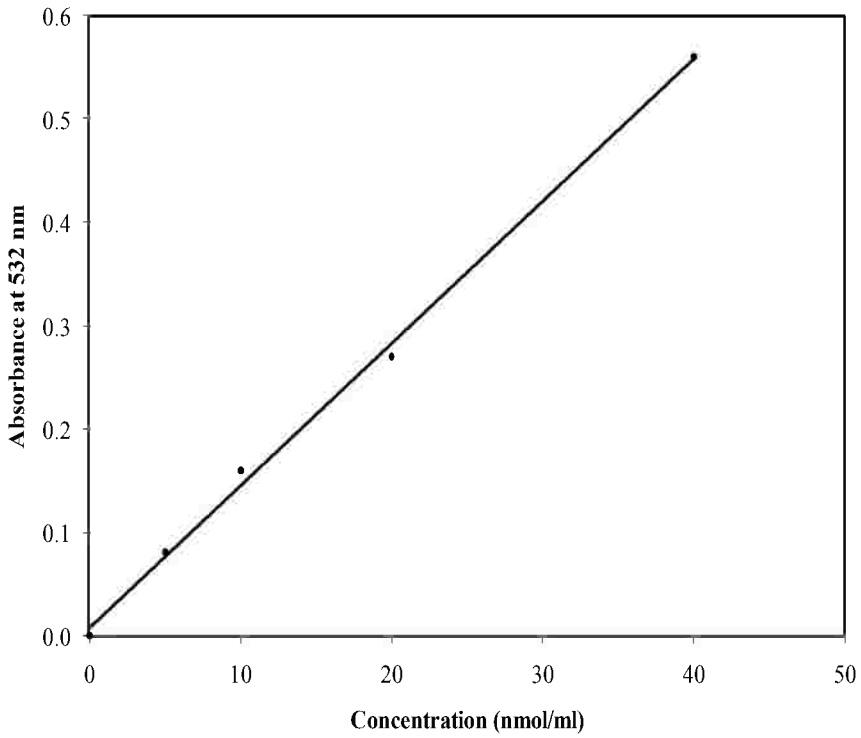

Figure (7): Standard curve of MDA

Determination of reduced glutathione (GSH):

Principle

The GSH was measured according to the method of Murphy et al [95].This method is based on the reductive cleavage of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) (DTNB), by SH group of glutathione to yield a yellow color with a maximum absorbance at 412 nm.

2GSH + DTNB → GSSG + 2 TNB (yellow colored chromophore)

Reagents:

- Trichloroacetic acid (TCA) and disodium salt of ethylene diaminetetraacetic acid (EDTA): disodium salt of EDTA (372 mg) and TCA (50 gm) were dissolved in one liter of distilled water.
- Solution A: 0.2 M prepared by dissolving 27.8 gm monobasic sodium phosphate (Merck Co, Germany), in one liter of distilled water.
- Solution B: 0.2 M prepared by dissolving 53.65 gm dibasic sodium phosphate (Merck Co, Germany), in one liter of distilled water.
- 0.1 M sodium phosphate buffer, pH 8: prepared by adding 26.5 ml of solution A to 473.5 ml of solution B and the volume was adjusted to one liter with distilled water.
- 0.1 M sodium phosphate buffer, pH 7: prepared by adding 195 ml of solution A to 305 ml of solution B and the volume was adjusted to one liter with distilled water.
- 0.01 M DTNB: prepared by dissolving 39.5 mg DTNB in 200 ml of 0.1 M sodium phosphate buffer, pH 7.

Assay procedure:

- 200 µl of the clear supernatant of the 20% tissue homogenate in TCA was added to 4.7 ml of 0.1 M sodium phosphate buffer pH 8, followed by 100 µl of 0.01 M DTNB and vortexed immediately for few seconds.
- The absorbance of the resultant yellow color was measured spectrophotometerically at 412 nm using within 25 minutes of addition of DTNB against a blank containing 200 µl distilled water instead of the sample.

Calculation

The concentration of GSH in the tissue samples was determined from a standard curve made by preparing serial dilutions of standard GSH in phosphate buffer pH 8 and treating them as samples. Results were subsequently expressed as µmol reduced glutathione/g tissue. Figure (8).

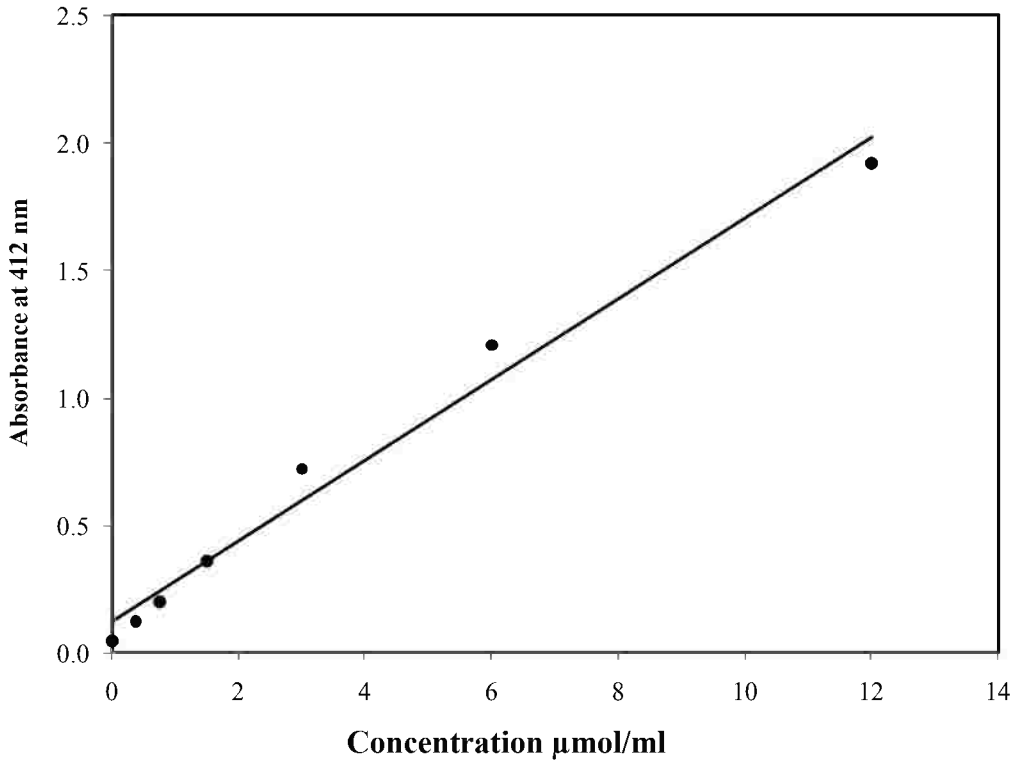

Figure (8): Standard curve of GSH

Determination of oxidized glutathione (GSSG):

The enzymatic method described by Griffith et al [96] was used to determine the oxidized glutathione content.

Principle:

This is a sensitive and specific enzymatic method which depends on the oxidation of GSH by 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) to yield GSSG and 5-thio-2-nitrobenzoic acid(TNB).

Reagents:

- 0.3 mM NADPH
- 6.0 mM DTNB
- 50 U/ml GSH reductase
- 2-vinyl pyridine
- 50% triethanolamine
- Standard GSH(5-100 nmol/ml)
- Standard GSSG(1-15 nmol/ml)

Assay procedure:

- Two µl of 2-vinyl pyridine was added to 100 µl of the sample with mixing.
- Six µl of 50% (v/v) triethanolamine was added to the side of the tube and the solution was vigorously shaked.
- The mixture was allowed to stand for 1 hour at room temperature.
- Aliquots of 0.1ml of mM DTNB,0.7 ml of 0.3 mM NADPH and 0.18 ml of distilled water were added ,mixed and incubated for 15 minutes.
- The reaction was initiated by the addition of 10 µl of glutathione reductase and the change in absorbance at 412nm per minute ΔA/min was measured.

Calculation:

The GSSG content in the samples were determined from a standard curve Figure (9). Results were subsequently expressed as µmol GSSG/g tissue.

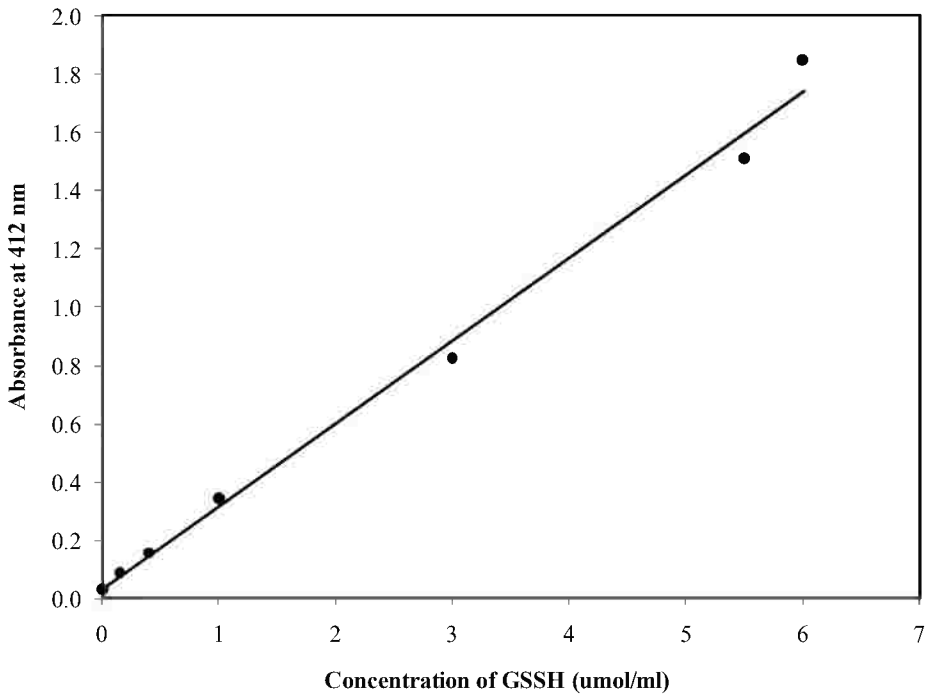

Figure (9): Standard curve for GSSG

Determination of total glutathione (tGSH):

Total glutathione was calculated using the following equation:

tGSH= GSH+ 2 GSSG

Determination of GSH/GSSG ratio:

The most widely used indicator of the redox state of the cells is the ratio of GSH to GSSG.

Statistical analysis of the data[1]

Data were fed to the computer and analyzed using IBM ***SPSS software package version 20.0.***[2] Quantitative data were described using mean and standard deviation. Comparison between the three studied groups were analyzed using F-test (ANOVA) and Post Hoc test (LSD). Significance of the obtained results was judged at the 5% level.

1. Kotz S, Balakrishnan N, Read CB, Vidakovic B. Encyclopedia of statistical sciences. 2nd ed. Hoboken, N.J.: Wiley-Interscience; 2006.
2. Kirkpatrick LA, Feeney BC. A simple guide to IBM SPSS statistics for version 20.0. Student ed. Belmont, Calif.: Wadsworth, Cengage Learning; 2013.