CHAPTER - 3

MATERIALS AND METHODS
3.1. Patients:

Fifty newly diagnosed breast cancer patients (ten at each stage), ranging in age from 32 to 65 years from Department of Oncology, Govt. General Hospital, Kurnool, Apollo Medical Centre, Kurnool (A branch of Apollo Hospitals, Hyderabad) and Gowri Gopal Hospitals, Kurnool, who had not undergone any previous treatment were chosen for this study. An equal number of age matched normal women served as control.

A data was collected from 130 women with newly diagnosed breast cancer from the Department of Oncology Govt. General Hospital, Kurnool and G.G Group of Hospitals, Kurnool, India. Patients were interviewed to obtain information on general demographic data, smoking, alcohol consumption, tobacco chewing, reproductive history, menopausal status and family history of breast cancer. Informed consent was obtained from all participants.

3.2. Blood Samples:

Blood was obtained by venous puncture in a sterile tube and was allowed to clot. Serum was separated by centrifugation at 1000g for 15 minutes. For plasma, blood was collected by venous arm puncture in a heparinised tube and plasma was separated by centrifugation at 1000g for 15 minutes. Blood samples obtained from breast cancer patients were analysed together with an equal number of age and sex matched normal healthy subjects.
3.3. Hemoglobin:

Hemoglobin was estimated by the cyanmethemoglobin method of Drabkin and Austin (1932). The basis of the method is dilution of blood in an alkaline solution containing potassium cyanide and potassium ferricyanide. Hemoglobin is oxidized to methemoglobin which combines with cyanide to form cyanmethemoglobin. The absorbance of the solution was measured at 540 nm. Values are expressed as grams / dl.

Reagents:

1. Drabkins reagent: 0.05g potassium cyanide, 0.02g of potassium ferricyanide and 1g of sodium carbonate in 1 liter of distilled water.

2. Cyanmethemoglobin standard solution (10g/dl)

Procedure:

To 0.02 ml of blood, 5.0 ml of Drabkins reagent was added, mixed well and allowed to stand for 10 minutes and absorbance was measured at 540 nm along with standard solution of cyanmethemoglobin against a blank containing 5.0 ml of the reagent in ELICO spectrophotometer.

3.4. Blood Sugar:

The blood sugar content was estimated by the method of Nelson Somogyi (1952). The reducing sugars on heating reduce the copper from the cupric to cuprous state. Cuprous oxide reduce molybdic acid to molybdenum blue. The absorbance of the solution was measured at 620 nm. Values are expressed as mg/dl.
Reagents:

1. Alkaline copper tartrate:

   Solution A: 2.5 g sodium carbonate, 2 g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20 g sodium sulphate in 100 ml distilled water.

   Solution B: To 15 g copper sulphate in distilled water, 0.05 ml sulphuric acid was added. The volume was made up to 100 ml.

2. Arsenomolybdate reagent: 2.5 g of ammonium molybdate was dissolved in 45 ml distilled water. 2.5 ml of sulphuric acid was added to this and mixed well. Then 0.3 g of disodium hydrogen arsenate dissolved in 25 ml distilled water was added and incubated at 37°C for 24 hours.

3. Standard Glucose Solution:

   100 mg of glucose was dissolved in 100 ml of water in a standard flask. 10.0 ml of the stock was diluted to 100 ml to get a working standard containing 100 μg/ml.

4. Zinc sulphate 5%

5. Barium hydroxide 0.3 N

Procedure:

To 0.1 ml of blood, 3.5 ml of distilled water, 0.2 ml of 0.3 N barium hydroxide and 0.2 ml of 5% zinc sulphate were added, mixed thoroughly and centrifuged. To 1.0 ml of supernatant, 2.0 ml of the alkaline copper solution was added and heated in a boiling waterbath for 10 minutes, cooled quickly for one minute. To this, 1.0 ml of the arsenomolybdate reagent was added and the volume was made up to 10 ml with water. The intensity of blue colour...
developed was read at 620 nm against blank in ELICO Spectrophotometer. Standards in the range of 20-100 µg were treated similarly.

3.5. Serum Copper:

The copper content was estimated by sodium diethyldithiocarbamate method (Eden & Green, 1940; Ventura & King, 1951). This method is based on the reaction of copper with sodium diethyl dithiocarbamate to form a golden yellow coloured complex, which is measured at 440 nm.

Reagents:
1. 2.0 N Hydrochloric acid
2. 20% Trichloroacetic acid (W/V)
3. 6% Sodium pyrophosphate
4. Saturated solution of sodium citrate
5. Ammonium hydroxide, diluted 2:1 with water (21% NH₃)
6. 0.1% Sodium diethyldithiocarbamate
7. Standard Copper Solution: The standard copper solution was prepared by dissolving 0.398 g of copper sulphate in distilled water, 0.1 ml of sulphuric acid was added to this solution and the volume was made up to one litre with distilled water. 1.0 ml of stock standard solution was diluted to 100ml to get a working standard containing 1 µg/ml.
Procedure:

To 1.0 ml of serum, 1.0 ml of 2.0 N hydrochloric acid was added and mixed thoroughly. To this, 1.0 ml of 20% trichloroacetic acid was added, mixed well and then centrifuged for 30 minutes at 3000 rpm. To 2.5 ml of supernatant, 0.2 ml of sodium pyrophosphate, 0.2 ml of sodium citrate, 0.4 ml of ammonium hydroxide were added and mixed well. 1.0 ml of 0.4% sodium diethyl dithiocarbamate solution was added and mixed thoroughly for few minutes. The absorbance of the solution was measured at 440 nm. Standards in the range of 0.5 – 2.5 μg were treated similarly along with a blank containing 2.5 ml of distilled water instead of 2.5 ml supernatant. Values are expressed as mg/dl serum.

3.5. Serum Iron :

Iron was estimated by Wong’s method (Silverton & Anderson, 1961) This method is based on the fact, ferric iron gives a red colour with potassium thiocyanate.

Reagents:

1. 6 N Hydrochloric acid

2. 20% Trichloroacetic acid

3. Nitric acid (Specific gravity 1.41)

4. 20% Potassium thiocyanate

5. Ether : Amyl alcohol mixture (1:2)
6. Standard Iron Solution: 702.2 mg Ferrous ammonium sulphate was dissolved in 100 ml distilled water. After the addition of 5 ml 1:1 Hydrochloric acid, the solution was made up to one litre and mixed thoroughly (100 μg Fe/ml). 10.0 ml of stock standard solution was diluted to 100 ml to get a working standard containing 10 μg Fe/ml.

Procedure:

1.5 ml of 6N hydrochloric acid was added to 3.0 ml of patient's serum, mixed well and incubated at 37°C for 60 minutes. 3.0 ml of 20% trichloroacetic was added with shaking and allowed to stand for 5 minutes. Then the tube was subjected to centrifugation and supernatant was separated. To the supernatant fluid, 0.1 ml of nitric acid and 1.0 ml of potassium thiocyanate were added and allowed to stand for 10 minutes, and extract into 5.0 ml of ether, amyl alcohol mixture, mixed thoroughly. To this a knife point of sodium sulphate was added, mixed well and allowed to settle. The absorbance of the solution was measured at 540 nm. Standards in the range of 1-5 μg were also treated similarly. Values are expressed as μg/dl serum.

3.6. Serum Mucoproteins:

Heat coagulable serum proteins are removed by means of perchloric acid, mucoproteins, which remain in solution are precipitated by phosphotungstic acid. The amount of mucoprotein thus precipitated has been estimated by determining its tyrosine content using Folin ciocalteau reagent (Lown et al., 1951).
Reagents:

1. 0.85% Nacl
2. Perchloric acid 1.8 M
3. 5% Phosphotungstic acid
4. Perchloric acid 0.6 M
5. Folin ciocalteau reagent: Commercially available (Himedia) reagent was diluted in the ratio of 1:2 with dislitted water just before use.
6. 20% Sodium carbonate
7. Standard tyrosine solution: 10 mg of tyrosine was dissolved in 100 ml of 0.1 N hydrochloric acid.

Procedure:

0.5 ml of serum was added to 4.5 ml 0.85% Nacl and 2.5 ml of 1.8 M perchloric acid was added drop wise with shaking. After 10 minutes the solution was filtered through a whatman No. 50 filter paper. To 5.0 ml of filtrate, 1.0 ml of 5% phosphotungstic acid was added, mixed and centrifuged for 10 minutes at 2000 rpm. The precipitate was washed with 0.6 M perchloric acid and the supernatant was decanted. To this 1.0 ml of 20% sodium carbonate, 3.5 ml water and 0.5 ml of Folin ciocalteau reagent were added and kept in a water bath at 30°C for 15 minutes. The absorbance of the solution was measured at 680 nm. The standards ranging in 20-200 μg were also treated similarly. Values are expressed as mg/dl serum.
3.7. Serum Inorganic Phosphorus:

The content of Inorganic phosphorus was estimated by Fiske and Subbarow method (1925). This method is based on the reaction of phosphate with molybdic acid to produce phosphomolybdic acid, which is reduced by 1, 2, 4 – amino naphthosulphonic acid to give a blue colour.

Reagents:

1. 10% Trichloro acetic acid
2. Sulphuric acid 10 N
3. 2 % Amonium molybdate
4. 15% Sodium bisulphite
5. 20% Sodium sulphite
6. 0.25% 1,2,4 – Aminonaphthol sulphonic acid
7. Standard phosphate solution: 0.351 g of potassium dihydrogen phosphate was dissolved in water. 10 ml of 10 N sulphuric acid was added to this and the volume was made up to one litre with distilled water. 10 ml of this stock solution was diluted to 100 ml to get a working standard containing 0.008 mg phosphorus / ml.

Procedure:

To 2.0 ml of serum, 8.0 ml of 10% trichloroacetic acid was added, mixed and filtered. To 5.0 ml of filtrate, 1.0 ml of ammonium molybdate was added and mixed thoroughly. To this 0.4 ml of 1,2,4 – aminonaphthol sulphonic acid was added and the volume was made up to 10 ml with distilled
water. The absorbance of the solution was read at 680 nm. Standards in the range of 8-40 µg were treated similarly together with the blank containing 5.0 ml of distilled water instead of filtrate. Values are expressed as mg/dl serum.

3.8. Serum Uric Acid:

Uric acid was estimated by the method of Caraway (1963). This method is based on the reduction of phosphotungstic acid by uric acid in the presence of sodium carbonate to give blue colour which can be read at 660 nm.

Reagents:

1. Sodium tungstate 10%
2. Sulphuric acid 2/3 N
3. Sodium carbonate 10%
4. Phosphotungstic acid: 50g of sodium tungstate was dissolved in 400 ml of distilled water. To this 40 ml of 85% phosphoric acid was added, refluxed gently for two hours. The final volume was made up to 500 ml with distilled water. 1.0 ml of this solution was diluted to 10 ml with water just before use.
5. Uric acid standard solution: 100 mg of uric acid was dissolved in 100 ml distilled water. 1.0 ml of this stock uric acid solution was diluted to 100 ml to get a concentration of 10 µg/ml.
Procedure:

5.4 ml of sodium tungstate was added to 0.6 ml of serum, mixed thoroughly and centrifuged. To 3.0 ml of supernatant, 0.6 ml of sodium carbonate and 0.6 ml of dilute phosphotungstic acid were added, mixed and incubated at 25°C for 30 minutes. The absorbance of the solution was measured at 660 nm against blank. Standards in the range of 5-30 µg were also treated similarly. Values are expressed as mg/dl serum.

3.9. Sialic Acid:

The glycoproteins in plasma were precipitated, hydrolyzed and protein bound sialic acid was estimated by the method of Warren (1958).

Reagents:

1. Periodic Acid: 0.025 M in 0.1N H₂SO₄

2. Sodium meta arsenate 4% in 0.5N hydrochloric acid.

3. Thiobarbituric Acid: 144 mg thiobarbituric acid was dissolved in 10 ml of water. The pH of the solution was adjusted to 9.0 with 6N sodium hydroxide.

4. Acidified butanol

5. Standard sialic acid solution: 10 mg of n-acetyl neuraminic acid was dissolved in 100 ml of distilled water (100 µg/ml).

6. Preparation of hydrolysate: To 0.5 ml of plasma, 2.0 ml of alcohol was added and centrifuged. The supernatant was decanted and precipitate was
dissolved in NaOH and hydrolysed with acid to liberate protein bound carbohydrate components.

Procedure:

To 1.0 ml of the hydrolysate sample, 0.25 ml of periodic acid was added and incubated at 37°C for 30 minutes. 0.25 ml of sodium meta arsenate was added to it and mixed well. To this, 2.0 ml of thiobarbituric acid was added and the contents were heated in a boiling water bath for 6 minutes. After cooling, 5.0 ml of acidified n-butanol was added to extract the pink colour formed into butanol phase. The butanol phase was removed after centrifugation and the absorbance was measured at 540 nm. Standard solutions containing 10-50 µg of n-acetyl neuraminic acid were also treated similarly. Values are expressed as µg/dl plasma.

3.10. Serum Acid Phosphatase:

Acid phosphatase activity was determined by the King and Armstrong (1934) method. This method is based on the principle that, the amount of p-nitrophenol liberated in unit time by the action of acid phosphatase on p-nitrophenyl phosphate. The released P-nitro phenol is in yellow colour in alkaline medium, measured at 405 nm.

Reagents:

1. 0.05 M citrate buffer; pH – 4.8

2. p-nitro phenyl phosphate solution 5 mM

3. Sodium hydroxide 0.1 N
4. p-nitro phenol standard solution 100 μmol

Procedure:

To 2.0 ml of p-nitro phenyl phosphate solution, 0.1 ml of citrate buffer and 0.4 ml of serum were added, mixed and incubated for 30 minutes at 25°C. The reaction was stopped by the addition of 4 ml 0.1 N sodium hydroxide. The absorbance was measured at 405 nm. For control, the serum was added after the addition of sodium hydroxide. Values are expressed as n moles of PNP liberated / min / dl serum.

3.11. Serum Alkaline Phosphatase:

The alkaline phosphatase activity was also determined by the King and Armstrong method (1934).

Reagents:

1. Sodium carbonate buffer 0.1 M, pH 10.

2. p-nitro phenyl phosphate solution, 5 mM

3. p-nitro phenol standard solution, 50 μmol

Procedure:

0.95 ml of carbonate buffer, (0.1M, pH 10) was added to 4.0 ml of p-nitrophenyl phosphate solution. To this 0.05 ml of serum was added and mixed gently. The absorbance was measured at 405 nm, after incubation at 25°C for 30 minutes against blank. Standard solution containing 50-300 n moles of PNP were also treated similarly. Values are expressed as n moles of PNP liberated / min / dl serum.
3.12. Reduced Glutathione:

Total reduced glutathione content was measured by the method of Ellman (1959). This method is based on the development of a yellow colour when 5, 5-dithio bis-2-nitro benzoate (DTNB) is added to compounds containing sulphhydryl groups.

Reagents:

1. Phosphate buffer, 0.1 M, pH 7.4
2. Phosphate buffer, 0.1 M, pH 8.0
3. 4% Sulphosalicylic acid
4. 0.11 mM DTNB

Procedure:

0.5 ml of 4% sulphosalicylic acid was added to the 0.5 ml of serum and the denatured protein removed by centrifugation for 20 minutes. To 1.0 ml of the supernatant, 4.5 ml of 0.11 mM DTNB in 0.1 M phosphate buffer (pH 8) was added. After 15 minutes the absorbance at 412 nm was measured against blank. For standards, different amounts of hydrogen peroxide ranging from 20-100 μmoles were taken and proceeded as above along with blank containing water. Values are expressed as μg/dl plasma.

3.13. Glutathione Peroxidase:

The activity of glutathione peroxidase was determined according to the method of Rotruck et al (1973). A known amount of enzyme preparation was allowed to react with hydrogen peroxide in the presence of
reduced glutathione. After a specific time period, the remaining Glutathione content was measured by the method of Ellman (1959).

**Reagents:**

1. Phosphate buffer, 0.4 M, pH 7.0
2. Sodium azide solution, 10 mM
3. 10% Trichloro acetic acid
4. Ethylenediaminetetra acetic acid (EDTA), 0.4 mM
5. Hydrogen peroxide ($H_2O_2$), 0.2 mM
6. GSH, 2Mm
7. 0.2% erythrocyte lysate: Erythrocyte lysate was prepared by following the procedure of Beutler.

**Procedure:**

The reaction mixture in a total volume of 1.0 ml contained 0.2 ml of phosphate buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.2 ml of 0.2% erythrocyte lysate. To the reaction mixture 0.2 ml of reduced Glutathione and 0.1 ml of $H_2O_2$ were added and incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 ml of 10% TCA. After centrifugation, the supernatant was assayed for GSH content using DTNB as described by Ellman (1959). Values are expressed as µg of GSH consumed / min / gram Hb.
3.14. Catalase:

Catalase was assayed by the method of Sinha (1972). The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H\textsubscript{2}O\textsubscript{2} with the formation of perchloric acid as an unstable intermediate. The chromic acid is measured colorimetrically at 620 nm. The catalase preparation is allowed to split hydrogen peroxide for different periods of time. The reaction is stopped at different time intervals by the addition of dichromate acetic acid mixture and the remaining H\textsubscript{2}O\textsubscript{2} is determined colorimetrically.

Reagents:
1. 0.01 M phosphate buffer, pH 7.0
2. 0.2 M Hydrogen peroxide
3. Dichromate – acetic acid reagent: 5% potassium dichromate solution was mixed with glacial acetic acid in the ratio 1:3.

Procedure:

To 1.0 ml of the phosphate buffer taken in each of four test tubes, 0.1 ml of enzyme preparation (erythrocyte lysate) was added. To this 0.4 ml of hydrogen peroxide was added. The reaction was stopped at 15, 30 and 60 seconds by the addition of 2.0 ml of reagent. The tubes were boiled for 10 minutes, cooled and read at 620 nm. For standards, different amounts of hydrogen peroxide ranging from 20-100 \mu mol were taken and proceeded as above along with blank containing water. Values are expressed as \mu g of H\textsubscript{2}O\textsubscript{2} decomposed / min / gram Hb.
3.15. Ascorbic Acid:

Ascorbic acid was estimated by the method of Omaye et al (1979). Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketoglut aric acid. These products when treated with 2,4-dinitrophenyl hydrazine, form the derivative bis 2-4-dinitrophenylhydrazone, which undergoes rearrangement to form a product with absorption maximum at 520 nm. Thiourea provides a mild reducing medium that helps to prevent interference from non ascorbic acid chromogens.

Reagents:

1. 2, 4-Dinitrophenyl hydrazine, thiourea, copper sulphate reagent (DTC): 0.4g thiourea, 0.05 g copper sulphate and 3.0 g of DNPH in 100 ml of 9N H₂SO₄.

2. 10% TCA

3. 65% H₂SO₄

4. Standard ascorbic acid solution: 10 mg/100ml 5% TCA

Procedure:

1.0 ml of the plasma was mixed thoroughly with 1.0 ml of ice cold 10% TCA and centrifuged for 20 minutes at 3500 g. To 0.5 ml of the supernatant, 1.0 ml of DTC reagent was added and mixed well. The tubes were incubated at 37°C for three hours, 0.75 ml of ice cold 65% H₂SO₄ was added and the tubes were allowed to stand at room temperature for 30 minutes. A set of standards containing 10-50 µg of ascorbic acid was processed similarly along with a blank containing 0.5 ml of 10% TCA. The colour developed was measured at 520 nm.
Values are expressed as mg/dl plasma.

3.16. Lipid Peroxidation:

Lipid peroxidation was estimated as evidence by the formation of thiobarbituric acid reactive substances like malondialdehyde according to the method of Yagi (1978).

Reagents:
1. 10% TCA
2. 0.67% Thiobarbituric and (TBA)
3. 0.25 N HCl

Procedure:

To 0.2 ml of plasma, 1.5 ml of 10% TCA was added followed by 0.8 ml of TBA. To this 0.5 ml of HCl was added and incubated in a boiling water bath at 100°C for 15 minutes. After cooling and centrifugation, the absorbance of the supernatant was read at 535 nm. A reagent bank was prepared by using water instead of plasma. The extent of lipid peroxidation was expressed as n moles MDA formed/ml plasma.

3.17. Statistical Analysis:

The data for biochemical analysis are expressed as mean ± S.D. t-test was applied to determine the significance of various biochemical changes among the clinical stages (I, II, III and IV) and controls. P value < 0.05 was considered as significant.