MATERIALS AND METHODS

Study area: The study will be carried out among the healthy individuals and hypertensive patients attending to the OPD of General Medicine of Rama Medical College, Hospital and Research Centre, Mandhana Kanpur, U.P. Non hypertensive healthy group will be selected from the volunteers of Rama Medical College hospital & research Centre, Kanpur.

Study type: This will be a prospective study hospital based case control study.

Study duration: The study duration will be of one year.

Sample size: Sample size will be a total of 200 subjects divided into two groups cases and control.

Cases: 100 patient's diagnosed with hypertension.

Control: 100 healthy individuals without any history of hypertension. Both of the group will be between 30 to 60 yrs of age group.

Inclusion Criteria: 1. Diagnosed cases of hypertensive patients.
2. Age group between 30 to 60 years
3. Male patients are included in the study.

Exclusion Criteria:
1. Congestive heart failure/heart block
2. Hematological diseases
3. Infectious diseases
4. Female hypertensives
5. Cigarette smokers
6. Alcoholics/tobacco/gutka
7. Patients on antioxidants drugs.
8. Diabetes

**Ethical Consideration:** - All the study process will be started only after obtaining ethical approval from the institutional ethical committee. All the information about the participants will be also kept confidential.

**Study tool:** - A pretested preformed meeting the objectives of the study is prepared. The cases for the study will be selected in accordance with the above mentioned inclusion and exclusion criteria, the purpose of the study will be explained to the patients and informed consent will be obtained. The data is collected according to the preformed in terms of detailed history clinical examination and necessary investigations.

The study will include the anthropometric assessment and blood biochemical markers for the oxidative stress.

- Anthropometric assessment (Height, weight, Body mass index)
- Blood pressure measurement – Systolic blood pressure, (SBP) diastolic blood pressure (DBP)
- Determination of hemoglobin
- Determination of blood groups (A, B, AB, O)
- Estimation of oxidative stress markers –
  - Malondialdehyde
- Superoxide dismutase
- Catalase

**Lipid profile**

- Estimation of serum Triglycerides
- Estimation of serum Total Cholesterol
- Estimation of high density lipoprotein - Cholesterol
- Estimation of low density lipoprotein - Cholesterol

**Methods of estimation**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>Cyanmethemoglobin method (Drabkins et al; 1932)</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>Thiobarbituric acid test (Ohkawa et al; 1979)</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Mishra &amp; Fridovich (1972)</td>
</tr>
<tr>
<td>Catalase</td>
<td>Aebi (1984)</td>
</tr>
<tr>
<td>Serum Triglycerides</td>
<td>Trinder, 1969</td>
</tr>
<tr>
<td>Serum Total Cholesterol</td>
<td>Roeschalu's; 1974</td>
</tr>
<tr>
<td>Serum High Density lipoprotein Cholesterol</td>
<td>Phosphotungstate (Brustein et al; 1970)</td>
</tr>
<tr>
<td>Serum low density lipoprotein Cholesterol</td>
<td>Friedwald et al; 1972</td>
</tr>
</tbody>
</table>
ANTHROPOMETRIC ASSESSMENT

Height

The height of the patients will be recorded with the help of a measuring tape by sticking it to a wall and keeping them in standing position without footwears.

Weight

Weight of the patients will be measured with help of a weighing machine without footwears.

Age

Age will be recorded as for the information provided by the respondents.

Body-mass index (BMI)

BMI is used as a measure of obesity and it will be derived using the standard formula by dividing weight with height (kg/m²).

Blood pressure measurement

The assessment of systolic and diastolic blood pressure will be done by sphygmanometer.

DETERMINATION OF BLOOD GROUP

The blood group will be determined in all the patients by the use of monoclonal agglutinating antibodies (Dacie and Lewis; 2001).
Principle

This test is based on haemagglutination reaction. Human red blood cells possessing A and/or B antigen agglutinate with the corresponding antibody. Agglutination of red blood cells with anti-A and anti-B antibody indicates the presence of the corresponding antigen. Absence of agglutination of red blood cells with anti-A and/or anti-B is a negative test and indicates the absence of corresponding antigen. For Rh system a drop of blood will be tested with anti-D serum. If agglutination occurs then the blood is Rh positive and if there is absence of agglutination then it is Rh negative.

DETERMINATION OF HAEMOGLOBIN

The haemoglobin content of the erythrocytes will be determined by cyanmethaemoglobin technique (Drabkin's et al; 1932).

This method involves conversion of haemoglobin in blood to cyanmethaemoglobin.

Principle

In the presence of potassium ferricyanide at alkaline pH, haemoglobin and its derivatives (except sulfhemoglobin) are oxidised to methemoglobin. Methemoglobin so formed reacts with potassium cyanide to form cyanmethemoglobin, a red coloured complex which is measured colorimetrically. The color intensity is proportional to the hemoglobin of the blood sample.
ESTIMATION OF MALONDIALDEHYDE

Thiobarbituric acid (TBA) test is used to estimate lipid peroxide content in plasma/serum by the method of Ohkawa (Ohkawa et al.; 1979).

Principle

In this one molecule of lipid peroxide, unsaturated conjugated dienes, hydroperoxides and malondialdehyde (MDA) reacts with two molecules of TBA with the production of a pink colour pigment of thiobarbituric acid reactive substances (TBARS) having an absorption maximum at 532-535 nm.

ENZYME ASSAYS - SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase activity will be determined by using spectrophotometric method of Mishra and Fridovich (McCord and Fridovich; 1972).

Principle

This procedure is based on the inhibition of a superoxide driven reaction (oxidation or reduction) by coupling with a superoxide detector i.e. the ability of superoxide dismutase to inhibit the reduction of nitro-blue-tetrazolium (NBT) by superoxide anion, or there is a competition for superoxide anion between superoxide dismutase and indicator molecule (NBT) that reacts avidly with the radical to produce a measurable change in absorbance. The reaction taking place are:

(a) Phenazine methosulphate (PMS) + NADH → Superoxide anions
(b) 

Superoxide anions + NBT → Formazan (insoluble purple blue colour).

Superoxide dismutase inhibits or competes reaction (b). Acetic acid was found to arrest the formazan formation when acetic acid was added prior to NADH addition, reaction did not initiate.

The intensity of purple blue colour correlate with the amount of formazan formation in NADH- phenazine methosulphate-nitrobluetetrazolium reaction in presence of superoxide anions.

The unit of enzyme activity is defined as the amount of enzyme required to inhibit the optical density at 560 nm of NBT reduction by 50% in one minute under assay conditions.

**CATALASE**

Catalase activity will be determined using spectrophotometric method of Aebi (Aebi; 1984).

** Principle**

In the ultraviolet range hydrogen peroxide shows the absorption maxima at 240 nm. The decomposition of hydrogen peroxide can be followed directly by the decrease in optical density at 240 nm. The difference in extinction per unit time is a measure of the catalase activity.
One unit of enzyme activity is defined as the amount of enzyme source required to decompose one micro mole of hydrogen peroxide per minute on pH 7.0 at 25°C.

LIPID PROFILE - ESTIMATION OF SERUM TRIGLYCERIDES

Triglycerides circulate in blood as complexes with protein molecules called lipoproteins. The serum triglyceride will be determined by the enzymatic method, employing lipoprotein lipase, glycerol kinase, glycerol phosphorase oxide (GPO) and peroxidase (Trinder; 1969).

Principle

Triglycerides are determined after enzymatic hydrolysis with Lipases. Peroxidase catalyses the conversion of hydrogen peroxide, 4-amino antipyrine and N-Ethyl, N-Sulfopropyl, N-anisidine (ESPAS) to a coloured quinonemine complex measurable at 540 nm.

Triglycerides \( \xrightarrow{\text{Lipase}} \) Glycerol + Fatty acids

Glycerol + ATP \( \xrightarrow{\text{Glycerol Kinase}} \) Glycerol-3-Phosphate

Glycerol-3 phosphate + O\(_2\) \( \xrightarrow{\text{GPO}} \) Dihydroxyacetone phosphate

H\(_2\)O\(_2\) + 4-amino antipyrine + ESPAS Purple Quinoneimine Complex + H\(_2\)O + HCl
ESTIMATION OF SERUM TOTAL CHOLESTEROL

The concentration of serum total cholesterol will be determined by modified Roes Chalu’s method (Roes Chalu’s; 1974).

Principle

Cholesterol esterase converts cholesterol esters to cholesterol and fatty acid. Cholesterol oxidase (CHOD) and peroxidase (POD) catalyses the conversion of cholesterol and hydrogen peroxide to red colour quinoneimine dye.

Absorbance of quinoneimine measured at 505 nm is proportional to cholesterol concentration in the specimen.

\[
\text{Cholesterol esters} \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{Fatty acid}
\]

\[
\text{Cholesterol} + \text{O}_{2} \xrightarrow{\text{CHOD}} \text{Cholesterol-4-en-3-one} + \text{H}_{2}\text{O}_{2}
\]

\[
2\text{H}_{2}\text{O}_{2} + \text{Phenol} + 4\text{-amino antipyrine} \xrightarrow{\text{POD}} \text{Red Quinoneimine Dye} + 4\text{H}_{2}\text{O}
\]

ESTIMATION OF HIGH DENSITY LIPOPROTEIN CHOLESTEROL

The cholesterol in high density lipoprotein will be determined by phosphotungstate method (Brustein et al; 1970).

Principle

Low density lipoprotein and very low density lipoprotein present in the serum is chemically precipitated with sodium phosphotungstate in presence of magnesium chloride. The high density lipoprotein remains in the supernatant
and total cholesterol content in it is estimated by the method described earlier by Roes Chalu’s; 1974.

The cholesterol content in very low density lipoprotein and low density lipoprotein were deduced by applying the Friedwald’s formula (Friedwald et al; 1972).

\[
\text{VLDL cholesterol} = \text{serum triglycerides/5}
\]

\[
\text{LDL cholesterol} = \text{total cholesterol} - (\text{VLDL cholesterol} + \text{HDL cholesterol}).
\]

**Sample size calculation and statistical analysis**

Sample size has been calculated in order type – I and II error assuming minimum power 80% and 95% significance level.

Formula: 

\[
\frac{2(P)(1-P)(Z^2 \beta + Z^2 \alpha / 2)}{d^2}
\]

- \(P\) = incidence of the disease
- \(q\) = \((1-P)\)
- \((P1-P2)^2\) or \(d^2\) is the difference which we want to detect at a specified power and level of confidence
- \(Z_{\beta}\) = Power of statistical test we want to be maximum 80% for which is \(Z_{\beta}\) is 0.84
- \(Z_{\alpha/2}\) = is the level of confidence we have chosen 95% confidence in this \(Z_{\alpha/2}\) = 1.96